High Complexity in the Expression of the B′ Subunit of Protein Phosphatase 2A<sub>0</sub>

EVIDENCE FOR THE EXISTENCE OF AT LEAST SEVEN NOVEL ISOFORMS*

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Protein phosphatase 2A (PP2A)<sup>1</sup> is one of the major serine/threonine protein phosphatases present in the cell and is involved in the control of many cellular functions and metabolic pathways (reviewed by Cohen (1989), Mumba and Walter (1993), DePaoli-Roach et al. (1994), and Mayer-Jaekel and Henmings (1994)). The Ser/Thr protein phosphatases, with the exception of PP2C, consist of multimeric structures. Their catalytic subunit associates with specific proteins, which serve as targeting/regulatory subunits and play substantial roles in the control of phosphatase activity.

PP2A is a family of holoenzymes containing a common core of a 36-kDa catalytic (C2) subunit and a 63-kDa A subunit associated with a variety of regulatory B-subunits (B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub>) to form the trimeric PP2A<sub>A</sub>, PP2A<sub>B</sub>, and polycation-stimulated protein phosphatase M, respectively (Tung et al., 1985; Waekens et al., 1987; Mumba et al., 1987; Zolniewicz et al., 1994). Takeda and co-workers (Usui et al., 1988) also isolated from human erythrocytes a PP2A form that contained a polypeptide of 74 kDa associated with the A-C2 core. Molecular cloning has identified in mammals two isoforms each of the C2 (da Cruz e Silva and Cohen, 1987; Green et al., 1987; Stone et al., 1987) and the A (Walter et al., 1989, 1990; Henmings et al., 1990) subunits, which are evolutionarily highly conserved.

The B-subunit is the most diverse and contains in various species, B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub>, of 52, 53, and 74–130 kDa, respectively. The B subunit, associated with PP2A<sub>A</sub>, and the B′ subunit, associated with PP2A<sub>B</sub>, appear to be structurally unrelated based on peptide mapping (Tung et al., 1985) and immunoreactivity (Zolniewicz et al., 1994). Molecular cloning of the B subunit has identified three closely related isoforms, α, β, and γ (Healy et al., 1991; Mayer et al., 1991; Zolniewicz et al., 1994), that are more than 80% identical. Drosophila (Mayer-Jaekel et al., 1993; Uemura et al., 1993) and Saccharomyces cerevisiae (Healy et al., 1991) homologs have also been isolated. Cloning of the cDNA for the B′ subunit, constituent of the polycation-stimulated protein phosphatase M, predicts the existence of two, 72- and 130-kDa, alternatively spliced forms (Hendrix et al., 1993) that show no homology to the B subunit isoforms. The dimeric A-C2 phosphatase has also been found to be associated with polyoma virus middle and small tumor antigens and with SV40 small tumor antigen (Pallas et al., 1990; Walter et al., 1990).

The control of PP2A activity is not fully understood. Recent reports suggest that post-translational modifications of the C2 subunit, such as phosphorylation and carboxymethylation, might have regulatory function (Chen et al., 1992; Lee and...
Stock, 1993; Xie and Clarke, 1993; Turowski et al., 1995). Several findings also point to a key role of the A and B-subunits in the regulation of phosphatase activity. In vitro studies indicate that the substrate specificity is affected by the subunit composition (Imaoka et al., 1983; Cohen, 1989; Agostinis et al., 1992; Kamiyashita et al., 1992, 1994). Association of the regulatory subunits with C2 has been demonstrated to cause either increased or decreased activity toward different substrates (Agostinis et al., 1992; Ferrigno et al., 1993; Mayer-Jaekel et al., 1994).

Support for the involvement of the regulatory B-subunits in the control of specific cellular functions comes from several studies. Association of the SV40 and polyomavirus antigens with the dimeric PP2A by displacement of the B-subunits might subvert the function of the enzyme and contribute to cell transformation (for a review see Mumbly and Walter (1993)). Transient expression of SV40 small t antigen in CV-1 cells stimulated the mitogen-activated protein kinase pathway and cell growth, most likely through inhibition of PP2A (Sontag et al., 1993). Mutations of the B subunit were found to result in defective cytokinesis in S. cerevisiae (Healy et al., 1991) and in abnormal anaphase progression in Drosophila (Mayer-Jaekel et al., 1993). Although the mechanisms responsible for the defects are not completely understood, it appears that the B subunit targets the phosphatase to distinct cellular structures (Sontag et al., 1995) and/or confers substrate specificity (Mayer-Jaekel et al., 1994).

Thus, strong evidence from different experimental approaches and systems points to a key role of the B-subunits in the control of PP2A holoenzymes. The elucidation of their primary structure is a critical step toward understanding their function. In this paper we report the isolation and characterization of cDNA clones encoding at least seven isoforms of the B′ subunit of PP2A in the control of specific cellular functions. The cDNAs had an open reading frame with an ATG at position 38. These cDNAs contained an open reading frame with a stop codon close to the 3′ end but did not have a translational start ATG codon. The 3′-end regions of five out of the eight clones characterized (M 1-8, 1013 bp; M 1-9, 819 bp) were sequenced (Fig. 1). The single stranded cDNA was then amplified with 100 pmol each of oligonucleotide 1, 2, 3, or 4 as forward primer. The amplified products were separated on 1% agarose gel and visualized by ethidium bromide staining.

Amplification of Rabbit Skeletal Muscle mRNA—Degenerate oligonucleotides were synthesized based on the amino acid sequence of four B′ subunit peptides: oligonucleotides 1, 2, 3, and 4 (5′-TT(T/C)CTG(T/A)G(A/T)C(T/G)(A/G)C(T/G)-3′). The single stranded cDNA was then amplified with 100 pmol each of oligonucleotide 1, 2, 3, or 4 as forward primer. The amplified products were separated on 1% agarose gel and visualized by ethidium bromide staining.

Isolation and Sequence Analysis of cDNA Library Clones—The 641-bp PCR fragment 3′-translated labeled by the random hexamer priming method (Feinberg and Vogelstein, 1983) was used for initial screening of 160,000 independent recombinants from an unamplified random primed rabbit skeletal muscle cDNA library (Zhang et al., 1989). Positive clones were plaque purified by consecutive screenings, and the cDNA inserts were subcloned into pTZ19U, M 13 mp18, and M 13 mp19 vectors for sequencing.

The initial screening of the rabbit skeletal muscle cDNA library with the 641-bp PCR fragment identified 38 positive clones. Fifteen clones were rescanned, and nine held positive after plaque purification. Four were fully characterized (M 2-1, 1338 bp; M 5-1, 1125 bp; M 1-1, 877 bp; and M 6-2, 1157 bp; Fig. 1), and five were found to overlap with the cDNA insert. Nucleotide sequence analysis of the clone showed 70% identity to the MPCR sequence. The four clones fell into two groups, comprising M 2-1 and M 5-1 and the other comprising M 1-1 and M 6-2 clones, indicating the existence of at least three isoforms. The isoforms encoded by M 2-1/5-1, termed α, and by M 1-1/6-2, termed β, shared 78 and 92% identity, respectively, with each other and with the cDNA insert. The combined α cDNAs contained an open reading frame with a stop codon close to the 3′ end but did not have a translational start ATG codon. The β cDNAs had an ATG start codon but no stop codon.

In order to obtain the complete coding sequences, the 5′ end 367-bp EcoRI–BglII and the 3′ end 329-bp Nael–EcoRI fragments of the α isoform, M 2-1 cDNA were recut with the unique restriction sites to identify the 5′ and 3′ ends. The 5′ end sequencing was performed using a T7-oligo-dAT primer at 3′ end with oligonucleotides 1 and 2, and the 3′ end sequencing was performed using the XbaI–EcoRI fragments of M 6-2B′ cDNA. Fifteen positive clones were identified, only one of which, M 8-68B′ j1 (1514 bp), extended the 5′ and 3′ ends (Fig. 1). This cDNA had an open reading frame with an ATG at position 24, immediately preceded by a stop codon, but no stop codon was present at the 3′ end. Therefore the 5′ 428-bp EcoRI–PstI, the 3′ 304-bp XmnI–EcoRI, and the 3′ 426-bp XbaI–EcoRI fragments of M 8-68B′ j1 were used for screening additional 120,000 recombinants of the rabbit skeletal muscle cDNA library. Sequence analysis revealed that the 3′ end regions of five out of the eight clones characterized (M...
19-1B \(\beta 2\), 316 bp; M 10-1B \(\beta 3\), 620 bp; M 17-2B \(\beta 3\), 922 bp; M 13-1B \(\beta 4\), 515 bp; and M 5-1B \(\beta 4\), 1502 bp) diverged from the M 8-6B \(\beta 1\) at different positions (Fig. 1 and 3). The subtypes of the \(\beta\) isofrom of the B subunit were termed \(\beta 1\), \(\beta 2\), \(\beta 3\), and \(\beta 4\).

320,000 additional recombinant \(\lambda\) phages were also screened with the radiolabeled 641-bp M PCR fragment in order to isolate \(\gamma\) isotype cDNA clones. Two overlapping clones were identified, M 20-1B \(\gamma 9\) (956 bp) and M 23-1B \(\gamma\) (956 bp) (Fig. 1). Although there are 11 differences (3 G/A and 8 T/ C) between the nucleotide sequences of the 918 bp overlapping portion of the two clones, their deduced amino acid sequences are identical, suggesting that they represent all-inic forms. The M 23-1B \(\gamma\) clone is identical to the M PCR in the overlapping region, except for some differences in the primer regions. These cDNA clones did not contain the complete coding sequence, and rescreening of the rabbit skeletal library failed to provide additional sequences. Because Northern analysis (see below) had indicated that the \(\alpha\) and \(\gamma\) isoforms were most abundant in brain, 220,000 recombinants from a \(\lambda\)gt10 rabbit brain oligo\(dT\)-primed cDNA library (Clontech Laboratories Inc.) were screened with the 641-bp M PCR fragment, the 536-bp M 5-4B \(\gamma\), the 423-bp M 1-8B \(\gamma\), the 773-bp SspI–EcoRI fragment of M 2-1B \(\gamma\), the 505-bp EcoRI–XbaI fragment, and the 665-bp XbaI–EcoRI fragment of M 8-6B \(\beta 1\). Several positive clones were isolated, eight of which encoded \(\beta\) \(\gamma\) and two of which coded for a distinct isoform, termed \(\delta\) (Fig. 1).

Amplification of DNA Fragments Spanning the \(\beta\) \(\gamma\) and B' X Clones—First-strand cDNA was synthesized from 5 \(\mu\)g of rabbit skeletal muscle and brain total RNA using the reverse primer 5'-CCCTGTCTGCACCAT-GCC-3' (nucleotides 636–652 of clone BR 6-1B \(\gamma\)). The PCR reactions were performed in which the dimeric phosphatase, in the absence or the presence of 0.2 M NaCl, was separated by SDS-PAGE and initially electroblotted onto a polyvinylidene difluoride membrane. NH\(_2\)-terminal sequencing of the intact B' polypeptide was not successful, indicating that the NH\(_2\)-terminus of the protein was probably blocked. In situ tryptic digestion, purification, and microsequencing of the released peptides provided sequences for nine B' subunit tryptic peptides, EVMFLGELE, FLGLR, FLESEOFQPS, TLPPDSNEDFGEELE, IQEIP, XQLEQ-TYPEV, XFMEMN, XXNNXIFR, and XPXPPGPPPSQ, covering a total of 81 residues. Comparison of these amino acid sequences with the predicted protein sequence of the B subunit isoforms of PP2A (Mayer et al., 1991; Healy et al., 1991; Zolnierowicz et al., 1994) and the B' subunit of polycation-stimulated protein phosphatase M (Hendrix et al., 1993) did not reveal any significant homology.

RESULTS

1. Isolation and Sequence Analysis of Protein Phosphatase 2A0 B' Subunit Peptides—The PP2A0 B' subunit was purified from rabbit skeletal muscle as previously reported (Zolnierowicz et al., 1994). The three subunits, A (61.5 kDa), B' (52.5 kDa), and C (36 kDa), were separated by SDS-PAGE and initially electroblotted onto a polyvinylidene difluoride membrane. NH\(_2\)-terminal sequencing of the intact B' polypeptide was not successful, indicating that the NH\(_2\)-terminus of the protein was probably blocked. In situ tryptic digestion, purification, and microsequencing of the released peptides provided sequences for nine B' subunit tryptic peptides, EVMFLGELE, FLGLR, FLESEOFQPS, TLPPDSNEDFGEELE, IQEIP, XQLEQ-TYPEV, XFMEMN, XXNNXIFR, and XPXPPGPPPSQ, covering a total of 81 residues. Comparison of these amino acid sequences with the predicted protein sequence of the B subunit isoforms of PP2A (Mayer et al., 1991; Healy et al., 1991; Zolnierowicz et al., 1994) and the B' subunit of polycation-stimulated protein phosphatase M (Hendrix et al., 1993) did not reveal any significant homology.

2. Cloning of the Regulatory B Subunit of PP2A0—Approximately 8 \(\mu\)g (80 pmol) of rabbit skeletal muscle dimeric PP2A (A-C2) was incubated in the presence or the absence of -1 \(\mu\)g (15 pmol) of purified His-tagged B at 4°C for 1 h in Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 mM NAD, 16.7 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride. Approximately 50 mg of Ni\(^{2+}\)-NTA-agarose were added to the sample and stirred slowly for 1 h at 4°C. The resin was then packed into a pipette tip column and washed with 25 ml Tris-HCl (pH 7.5), 10 ml imidazole, and 0.2 mM NaCl. The bound proteins were eluted with 25 ml Tris-HCl (pH 7.5), 0.03 mM EGTA, 0.2 mM NaCl. Aliquots were analyzed by SDS-PAGE and Western immunoblotting. Control experiments were performed in which the dimeric phosphatase, in the absence of B', was applied to the Ni\(^{2+}\)-NTA-agarose, and the column was developed as above.

Western Blot Analysis—Protein samples were separated on 9% SDS-PAGE and electroblotted onto nitrocellulose as described in Tang et al. (1991). Filters were incubated for 2 h with antibodies to the bovine heart PP2A0 B' subunit (Mumby et al., 1987), the 314–324 peptide of A0 (Zolnierowicz et al., 1994), and the C2 (Mumby et al., 1985). Bound antibodies were detected with \(^{125}\)I-protein A (0.2 \(\mu\)Ci/ml) and autodigestion.
two clones had overlapping, identical regions, but the 225–696 nucleotide region of M 1-7B' α cDNA was not present in the M 7-1B' α (Fig. 1). This deletion eliminated the initiator codon. The significance of the deletion is not clear at this time. It is possible that it represents an alternatively spliced variant of B' α.

Analysis of B'β cDNA Clones—Rescreening of the muscle library with β specific cDNA probes led to isolation of clones that diverged at different positions in their 3' end regions (Figs. 1 and 3), most likely generated by alternative splicing. The subtypes of the β isofrom of the B' subunit were termed β1, β2, β3, and β4. Their deduced amino acid sequences are identical up to residue 442 but diverge in their COOH termini (Fig. 3) at positions in the nucleotide sequence corresponding to potential splice sites (Fig. 3; Senapathy et al., 1990). The β4 subtype starts to diverge at nucleotide 1350 of the M 8-6B' β1 and contains a stop codon 21 nucleotides downstream from the point of divergence. Combination of the common region and β4 cDNAs provides a 1509-nucleotide sequence containing an open reading frame of 1348 bp, encoding a 52.5-kDa protein that shares 64% identity and 74% homology (Table I) with the α isoform. The 139-bp 3' untranslated region contains a polyadenylation signal 21 bp upstream of the 3' end, which may contain the beginning of the poly(A) tail. This was confirmed by amplification of the 3' end by reverse transcriptase PCR, which generated a 260-bp fragment identical to the 3' region of the β4 cDNAs except for the presence of a stretch of 17 adenines at the end.

The β2 and β3 clones start to diverge from β1 and from each other at nucleotide 1467 (Fig. 3). The β2 cDNA has a stop codon 16 bp downstream, and the β3 cDNA has a stop codon 130 bp downstream. Combination of the common region of the B'β cDNAs with the β2 or β3 isoforms yields sequences of 1458 or 1572 nucleotides, encoding proteins with predicted molecular weights of 56,500 or 60,900, respectively. Interestingly, the divergent region of β3 contains a putative bipartite nuclear localization signal RKTVDSEARQAQQKDPPKK (Dingwall and Laskey, 1991; Robbins et al., 1991).

Characterization of B'γ and δ cDNAs—Screening of the muscle library did not provide complete coding sequences for the γ isoform of B' (Fig. 1 and 5). However, screening of a rabbit brain library resulted in isolation of several cDNA clones. The nucleotide sequences of the 3' end region of these cDNAs were identical to those of the rabbit skeletal muscle M 23-1B' γ and the amplified M PCR fragment (Fig. 1). Restriction mapping and partial sequence analysis revealed that the 5' region of the rabbit brain γ clones were different and showed no homology to the corresponding regions of the other isoforms. Previous screening of the same library had suggested that this brain library might contain concatamers of unrelated cDNA fragments (Zolnierowicz et al., 1994). Nevertheless, the BR 6-2B' γ brain cDNA (Fig. 1) contained an open reading frame of 1308 bp. An ATG codon, at nucleotide 289, is preceded 30 bp upstream by an in frame stop codon. Another rabbit brain clone, BR 6-1B' X, had a 445-bp open reading frame ending with a stop codon. Joining the BR 6-2B' γ and BR 6-1B' X cDNAs at the EcoRI site did not change the reading frame. Reverse transcriptase PCR amplification from rabbit skeletal muscle and brain RNA using as primers oligonucleotides derived from the BR 6-2B' γ and the BR 6-1B' X cDNAs yielded a 1045-bp product (Fig. 1, B' γ-X). This DNA fragment contained an internal EcoRI site, confirming that the two cDNA clones pertained to the same mRNA. Combined sequences of B'γ and X cDNAs provided a 1758-bp open reading frame encoding 586 amino acids with a predicted molecular mass of 68 kDa (Fig. 4). The muscle and brain B'γ isoforms are identical (Fig. 5) and display higher homology to the B'β isoforms (77–82%) than to the B' α (61%) (Table I). In the NH2-terminal region, starting at amino acid 21, there is an 8-fold glutamine-proline repetition, and in the COOH-terminal 532–548 residues there is a putative bi-
partite nuclear localization signal.

Screening of the rabbit brain library identified two additional clones, BR 6-1B and BR 8-1B, that are identical at the 3' end but differ at the 5' end (Fig. 1). This region might be the result of the presence of concatamerization of brain cDNAs in the library (Zolnierowicz et al., 1994). A putative translation start codon is present in a reasonable (AGTAGGGATATG) consensus sequence for initiation (Kozak, 1989). The 495-bp partial coding region encodes 165 amino acids that share 64, 55, and 56% identity with the corresponding regions of the α, β, and γ isoforms, respectively (Fig. 5 and Table I).

### Tissue Distribution of the α, β, γ, and δ Isoform mRNAs—Northern analysis performed with total RNA isolated from several tissues and cell lines indicated a tissue-specific distribution of the B9 subunit isoforms (Fig. 6). The transcripts coding for Bα (3.0 kb) and Bγ (3.7 kb) are highly expressed in brain (Fig. 6, A and C). There are three distinct transcripts of 1.7, 2.2, and 4.3 kb coding for Bβ (Fig. 6B). The 4.3-kb transcript is most abundant in testis, followed by brain, heart, and skeletal muscle. The 1.7-kb transcript is more highly expressed in rabbit heart and spleen. The 2.2-kb transcript is detectable in heart, testis, and brain. Three transcripts of 3.9, 3.1, and 2.8 kb were detected for Bγ (Fig. 6D). The 3.9-kb transcript is most abundant in testis and brain followed by lung and L929.
The 3.1-kb transcript seems to be present exclusively in rat testis. The 2.8-kb message can be detected in several tissues but is more abundant in rabbit testis. A higher molecular weight transcript of 8.2-kb most prominent in brain was also observed.

Interaction of the Recombinant Bβ with the A-C2 Form of Protein Phosphatase 2A—Expression of Bβ in E. coli produced proteins of 53 or 55 kDa that were mostly insoluble. Growing the cells at lower temperature did not improve the solubility of these proteins.

**Fig. 3.** Nucleotide and deduced amino acid sequences of the rabbit skeletal muscle Bβ isoforms of PP2A0. Combined sequences of all the Bβ cDNA clones in Fig. 1 are shown. Single underlines indicate the potential splice sites where the B1, 2, 3, and 4 isoforms diverge. The double underline shows the polyadenylation signal, and the dots represent the stop codons.
Cloning of the Regulatory B' Subunit of PP2A0

The upper left half shows the nucleotide sequence identity, and the lower right half shows the identities of the deduced amino acid sequences among the rabbit B' subunit isoforms and human brain (HuBr, T09026), human myeloid (HuMy, D26445), mouse (Mus, L26793), mouse testis (MXT, X81059), RT51 (P06630), and ricin (RICC, D22057) present in GenBank. The letter followed by the numbers indicates the accession number for each sequence. Numbers in parentheses show the homology based on the following grouping of conservative amino acids: Val, Ile, and Leu; Phe, Tyr, and Trp; Asp and Glu; Asn and Gin; and Arg and Lys. The dash indicates that no overlapping region is present.

| Identity | B'α | B'β1 | B'β2 | B'β3 | B'β4 | B'γ | B'δ | HuBr | HuMy | Mus | MMT | RT51 | RICC |
|----------|-----|------|------|------|------|-----|-----|------|------|-----|-----|------|------|
|          | 58  | 58   | 57   | 58   | 59   | 52  | 91  | 61   | 67   | 53  | 55  | 65   |      |
| B'β1     | 62 (71) | 98   | 98   | 94   | 94   | 68  | 53  | 68   | 85   | 87  | 57  | 61   | 62   |
| B'β2     | 62 (71) | 99 (99) | 93   | 93   | 68   | 53  | 68   | 83   | 87   | 57  | 61  | 62   |      |
| B'β3     | 62 (71) | 97 (97) | 99 (99) | 92   | 69  | 53  | 68   | 91   | 87   | 60  | 56  | 62   |      |
| B'β4     | 64 (74) | 98 (98) | 98 (98) | 98 (98) | 69  | 53  | 68   | 82   | 87   | 50  | 58  | 62   |      |
| B'γ      | 61 (71) | 77 (83) | 77 (83) | 77 (83) | 82 (87) | 46  | 74  | 64   | 74   | 86  | 47  | 67   |      |
| B'δ      | 64 (76) | 55 (63) | 55 (63) | 55 (63) | 56 (63) | 75  | 64  | –    | –    | 48  | –   | –    |      |
| HuBr     | 100  | 84 (88) | 84 (88) | 84 (88) | 84 (88) | 84 (88) | 64  | 66   | –   | –   | 46  | 66   |      |
| HuMy     | 66 (74) | 91 (91) | 91 (91) | 99 (99) | 98 (98) | 76 (80) | 54 (63) | 83 (84) | 91   | 49  | 53  | 61   |      |
| Mus      | 79 (89) | 97 (97) | 97 (97) | 97 (97) | 97 (90) | 96   | –   | –    | 97 (97) | –   | 62  | 58   |      |
| MMT      | 28 (35) | 48 (59) | 50 (62) | 46 (58) | 58 (67) | 90 (90) | –   | –    | –   | –   | 30  | –    |      |
| RT51     | 53 (65) | 56 (66) | 56 (66) | 56 (66) | 56 (60) | –   | 36 (48) | 67 (78) | 56 (67) | 69 (82) | 15 (27) | 59   |      |
| RICC     | 64 (72) | 64 (74) | 64 (74) | 64 (74) | 64 (75) | –   | 66 (74) | 64 (76) | 50 (68) | 63 (73) |      |      |      |

**DISCUSSION**

The PP2A has been isolated in a variety of trimeric forms that differ in the associated B subunits. The 52-kDa and the 72-130-kDa constituents of the PP2A and the polycation-stimulated protein phosphatase M holoenzymes, respectively, have been previously cloned. In this paper we report the molecular cloning of cDNAs encoding the B' subunit of the previously characterized PP2A0 (Zolnierowicz et al., 1994). Screening of rabbit skeletal muscle and brain cDNA libraries has led to the isolation of at least seven cDNA isoforms that fall into four subgroups, termed α, β, γ, and δ. The β subgroup comprises four isoforms that appear to be generated by alternative splicing at the 3' end. Complete coding sequences have been obtained for five isoforms, α, β2, β3, β4, and γ (Fig. 5).

Eight of nine peptides isolated from the rabbit skeletal muscle protein were found to be identical or to share high homology with the predicted amino acid sequences of the B' isoforms (Fig. 5). Three of the peptides have sequences identical to those in the β isoform. It is possible that this was the predominant form in our PP2A0 preparation and that sequences identical to those of the other peptides would have been found if the complete coding region had been isolated. The lack of complete identity could also be explained either by errors in sequencing of the peptides or by the presence of a mixture of different isoforms in the purified phosphatase. Alternatively, not all the existing isoforms have been isolated. Nevertheless, the reactivity of the recombinant B'α with antibodies against the bovine heart B subunit, which we have shown to be of the B' form (Zolnierowicz et al., 1994), and the ability of the recombinant protein to associate with the A-C2 dimer, clearly indicate that the cloned cDNAs encode B' subunit isoforms.

The α encoding cDNAs predict a protein of 57.6 kDa. The start codon is preceded 30 bp upstream by an in frame stop codon. The presence of a deletion that eliminates the initiator codon in one of the cDNAs suggests that alternative splice variants of the α isoform may also exist. The β subgroup comprises four isoforms, β1, β2, β3, and β4, that most likely are generated by alternative splicing at the 3' end. All four contain an identical 442 amino acid region and diverge at their COOH termini. The nucleotide sequence around the points of divergence is in agreement with mammalian splice junction boundaries, AG/ GT for β2 and β4, AG/GC for β3, and AG/CA for β1 (Senapathy et al., 1990). The detection of three B'β transcripts by Northern analysis further supports the existence of multiple forms. The nucleotide sequence surrounding the first ATG (GGAGCTAGATGT) is in moderate agreement with the consensus sequence for initiation (Kozak, 1989). This putative start codon is immediately preceded by a stop codon. The next downstream potential initiation codon is at position 54. The nucleotide sequence (GGCAGGACTG) around this second ATG complies better with the consensus sequence for translational initiation, but at this time it is not clear which one is actually used.

Nucleotide and amino acid sequence analysis indicated that all seven isoforms share a high degree of homology (Fig. 5, and Table I). The α and β1, 2, 3, or 4 isoforms show ~58% nucleotide and ~62% amino acid sequence identity. The γ isoform
shares higher amino acid homology with the β isoforms (77–82%) than with the α (61%). Conversely, the δ is more similar to the α (64%) than the γ (56%) and the βs (55%). The homology is higher in the central regions and diverges most at the NH₂ and COOH termini (Fig. 3). Thus, one could speculate that the homologous region may be involved in interaction with the A and/or C₂ subunits, whereas the termini could confer specific properties to the holoenzyme. In support of this hypothesis, two of the isoforms, the γ and β3, contain the bipartite nuclear localization motif (K/R)(K/R)XXXXXXXXXXKXXKK (Dingwall and Laskey, 1991; Robbins et al., 1991). This sequence may be responsible for directing specific PP2A₀ holoenzyme forms to the nucleus. Indeed nuclear association of PP2A activity has been described (Jakes et al., 1986; Turowski et al., 1995), although the exact type of the enzyme is not known. The γ isoform also contains at the NH₂ terminus an 8-fold glutamine–proline (QP) repeat whose significance is not clear. Interestingly the 74-kDa polypeptide present in the PP2A holoenzyme isolated by Takeda and co-workers (Usui et al., 1988) is an alternatively spliced variant of B9g₂. Thus, the enzyme of Usui et al. (1988) belongs to the PP2A₀ family.

2 M. Takeda, personal communication.
Fig. 5. Alignment of amino acid sequences of different B' subunit isoforms. Deduced amino acid sequences of the rabbit skeletal muscle (RSM) and brain (RB) B' subunit isoforms, human myeloid cell cDNA (HUMORFY), and S. cerevisiae rac3 suppressor gene product, Rts1p, were aligned with the sequence of the B'' isoform. Identical amino acids are designated by small dots; dashed lines indicate gaps, and solid diamonds designate stop codons. The numbers in the Rts1p sequence indicate residues that were not included in the alignment. The peptide sequences obtained from the purified rabbit skeletal muscle (RSM B' Peptide) B' subunit are also shown. The underlined sequences indicate the putative nuclear localization signals.
The tissue-specific expression of the B' isoforms is also in keeping with the idea that different isoforms of the B' subunit may direct the enzymes to different cellular functions. The α and γ mRNAs are more abundant in brain. The 4.3-kb transcript of β is highly expressed in testis, whereas the 1.7-kb message is more abundant in heart and spleen. Testis, lung and brain express the highest level of δ mRNA.

Data base searches revealed no homology between the B' subunit isoforms and the other known B-subunits of PP2A, including the small and middle SV40 and polyoma virus tumor antigens. However, matches with several other nucleotide sequences present in GenBank were found. The protein sequence deduced from a 382-bp cDNA, T09026 (Adams et al., 1993), is identical to a region of B' if the T and the G at positions 339 and 373 of the EST clone are deleted. A human open reading frame, HUMORFY (D26445, 3702 bp), shares more than 90% identity with the B'β group at the level of amino acids (Table I). Comparison of the amino acid sequence of HUMORFY with the B'β3 revealed that the human clone contains a deletion corresponding to amino acids 442-480 of the B'β3, but the downstream sequences are almost identical. Interestingly, the B'β4 clone starts to diverge from the other three β isotypes at the position where the deletion in the HUMORFY starts, and β1, β2, and β3 diverge from each other at the point where the deletion ends (Fig. 5). These positions correspond to potential splice junctions. Therefore, it is possible that a fifth alternative spliced form of B'β exists.

Two mouse clones in the data base also share high homology with the B' isoforms. The 332-bp sequence of MUSF354A (L26793) codes for a protein that is 97, 79, and 90% identical with B'β, B'α, and B'γ, respectively, if minor changes are introduced. The other mouse clone, MMTEG271G (X81059), is 86 and 90% identical to the B'γ at the level of nucleotides and amino acids, respectively. These clones appear to represent the mouse β and γ isotype of B'. In addition a rice 419-bp cDNA clone, RICC102651 (D22057), was found to have 64-65% amino acid identity with the rabbit B' isoforms.

An especially interesting match was found with a S. cerevisiae gene (U06630), isolated as a high copy suppressor of roc3
Cloning of the Regulatory B′ Subunit of PP2A

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