Identification of a Novel Protein Phosphatase 2A Regulatory Subunit Highly Expressed in Muscle*

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Differential association of regulatory B subunits with a core heterodimer, composed of a catalytic (C) and a structural (A) subunit, is an important mechanism that regulates protein phosphatase 2A (PP2A). We have isolated and characterized three novel cDNAs related to the Bα subunit of bovine cardiac PP2A. Two human (Bα1 and Bα2) and a mouse (Bα3) cDNA encode for alternatively spliced variants of the Bα subunit. The deduced primary sequences of these clones contain 12 of 15 peptides derived from the purified bovine Bα subunit. Differences between the deduced sequences of the Bα splice variants and the cardiac peptide sequences suggest the existence of multiple isoforms of the Bα subunit. Comparison of the protein and nucleotide sequences of the cloned cDNAs show that all three forms of Bα diverge at a common splice site near the 3′-end of the coding regions. Northern blot and reverse transcription-polymerase chain reaction analyses revealed that the Bα transcripts (4.3–4.4 kb) are widely expressed and very abundant in heart and skeletal muscle. The expressed human and mouse Bα proteins readily associated with the PP2A core enzyme in both in vitro and in vivo complex formation assays. Immunofluorescence microscopy revealed that epitope-tagged Bα was localized in both the cytosol and nuclei of transiently transfected cells. The efficiency of binding of all three expressed proteins to a glutathione S-transferase-A subunit fusion protein was greatly enhanced by the addition of the Cα subunit. Expression of the Bα subunits in insect SF9 cells resulted in formation of AC-Bα heterotrimers with the endogenous insect A and C subunits. These results show that the Bα subunit, which is the predominant regulatory subunit in cardiac PP2A, is a novel protein whose sequence is unrelated to other PP2A regulatory subunits. The nuclear localization of expressed Bα suggests that some variants of the Bα subunit are involved in the nuclear functions of PP2A.

Protein phosphorylation is an essential mechanism regulating a wide variety of cellular processes. The coordinate activity of protein kinases and phosphatases is required for normal signal transduction. Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that has been implicated in the control of the cell cycle (1), growth and proliferation (2), DNA replication (3), viral transformation (4), and morphogenetic events (5). The PP2A holoenzyme is a heterotrimer composed of a 38-kDa catalytic (C) subunit, a 63-kDa structural (A) subunit, and a third subunit termed B or phosphatase regulatory (PR) subunit (6, 7). There are at least five distinct families of proteins that interact with and regulate the PP2A core enzyme. These include the Bα, Bβ, PR72 (Bα2), and α families of regulatory subunits and the small and middle tumor antigens of DNA tumor viruses (8).

The diversity of PP2A regulatory subunits suggests specific physiological roles for individual holoenzymes. Genetic studies have shown that disruption of the yeast homolog of the Bα subunit, CDC55, results in defects in budding and cytokinesis (9). Decreased expression of the Drosophila Bα subunit causes defects in mitosis, duplication of wing imaginal discs and is lethal in the late larval/early pupal stage of development (5, 10). Biochemical studies have demonstrated that the Bα subunits and tumor antigens affect the substrate specificity and activity of PP2A (11–13). Sensitivity to polyamines, polycations, and ceramide is also dependent on the type of Bα subunit associated with the AC core complex (13–15). These data support an emerging hypothesis that differential association of Bα subunits regulates PP2A function in vivo.

Molecular cloning has revealed diversity within the PP2A regulatory subunit families. Multiple isoforms (α, β, γ) of the Bα subunit and a splice variant of Bα have been isolated from mammalian sources (9, 16, 17). These proteins are 81–87% identical and diverge primarily at the amino termini. Two cDNAs encoding 72- and 130-kDa forms of the PR72 (Bα2) subunit were obtained from human heart and brain libraries (18). PR130 has an extension at the amino terminus that is generated either by alternative splicing or the use of an alternative promoter site. In this report, we describe the isolation and expression of novel cDNAs encoding human (Bα1 and Bα2) and mouse (Bα3) members of the Bα family of PP2A regulatory subunits. The recombinant subunits readily form heterotrimeric complexes with the AC core enzyme in vitro and in vivo. Transient expression of human Bα in mammalian cells revealed the presence of both cytoplasmic and nuclear populations of the regulatory subunits. The Bα subunits may be important for the localization/translocation of PP2A into the nucleus.

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5164
MATERIALS AND METHODS

Molecular Cloning of the Human B'-Subunit—PP2A (AC-B') was purified from bovine cardiac tissue, and partial amino acid sequence of the B' subunit determined as described previously (12). The peptides derived from the bovine cardiac B' subunit were used to search for similar sequences in the GenBank data base using the BLAST network service of the National Center for Biotechnology Information. This search revealed significant homologies between the peptide and a random cDNA from human KG1 cells (accession number D26445). The sequence of this cDNA was used for the design of oligonucleotide primers. An 854-bp fragment was amplified by polymerase chain reaction (PCR) as described previously (13) from a human umbilical vein epithelial cell (HUVEC) cDNA library (kindly provided by Drs. J. Battey and Mark Akeson, NIIDD) using a sense primer 5'-CTCAGTGCTTCTACCATCCCCAG-3' and an antisense primer 5'-TGGAAATGGTGACTTCACTTCT-3' (nucleotides 1605-1629) in which several bases were mutated (underlined) to generate an EcoRI restriction site. The thermal profile (94°C, 1 min; 50°C, 1 min; 72°C, 1 min) was carried out for 30 cycles. The PCR fragment was subcloned into pCR1 (Invitrogen) and the sequence was verified. Random priming of this fragment was used to generate a radiolabeled probe (10^6 dpm/µg) to screen the HUVEC cDNA library. Prehybridization and hybridization of filters containing 10^6 independent recombinants was performed at 65°C in 5 x SSC, 5 x Denhardt’s solution, 0.1% SDS, and 100 µg/ml herring sperm DNA. The filters were washed 4 times (5 min) in 2 x SSC, 0.1% SDS at room temperature, followed by a 20-min wash in 0.4 x SSC, 0.1% SDS at 65°C, and subjected to autoradiography at −20°C. Five positive clones (HB-2, 3, 5, 6, 7) were obtained as bands of plaque purification. Recovered plasmids from the HUVEC cDNA library was carried out according to the manufacturer’s protocol (Stratagene). The cDNAs were sequenced on both strands by the dideoxy nucleotide chain termination method (19).

Yeast Two-hybrid Screening—The A subunit of PP2A (20) was inserted into the Smal/SalI sites of pSA1-CHY2 to generate a fusion with the GAL4 DNA binding domain. Yeast transformed with this plasmid were used to screen a mouse T-lymphocyte cDNA library, constructed as fusions with the GAL4 activation domain (10^6 transformants), using the two-hybrid assay (21). Positive colonies were selected on Trp-, Leu-, and His- medium in the presence of 50 µg 3-amino-triazole and screened for β-galactosidase activity by the colony lift method (22). The activation domain pACT-fusion plasmids were extracted from the mouse T-lymphocyte cDNA library and used in binding assays with the β'-subunit of PP2A (13) was subcloned into the EcoRI/XhoI and Smal/SalI sites of pACTII, respectively. Ncol/BamHI fragments from the pRCMV-B' plasmids (see below) were subcloned into the appropriate sites in the GAL4 DNA binding domain vector, pSA1-CHY2. Expression of β-galactosidase activity in the two-hybrid assay was determined as described above (22).

Northern Analysis and Reverse Transcription PCR—A 1.5-kb EcoRI fragment (nucleotides 2501-4064 of B'-1 clone HB-7) was used to probe a mRNA blot of human tissues (Clontech). Prehybridization was carried out in 50% formamide, 10 x Denhardt’s solution, 5 x SSPE, 2% SDS, and 100 µg/ml herring sperm DNA at 42°C. Hybridization was carried out in the same solution with the radiolabeled (1.5 x 10^6 dpm/µg) EcoRI fragment for 16 h at 42°C. The filter was washed 2 times in 2 x SSC, 0.1% SDS at room temperature followed by a 20-min wash in 0.2 x SSC, 0.1% SDS at 50°C. The blot was subjected to autoradiography for 6 h at −80°C with an intensifying screen.

Poly(A)^+ RNA was isolated from mouse tissues, and first strand cDNA was synthesized as described previously (23). The mouse cDNA was used in a PCR with oligonucleotide primers corresponding to nucleotides 5'-CTCAGTGCTTCTACCATCCCCAG-3' and 1413-1436 (antisense 5'-CTCGGAGCTTGGGTGGTGGATGCC3') of B'-2. The thermal profile (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) was carried out for 30 cycles.

Construction of B'-Expression Plasmids—To facilitate the insertion of the HUVEC B'- cDNAs into various expression vectors, a short fragment (171-175) of cDNA for B'-1 (B'-2) was amplified by PCR and subcloned into pCR1. The antisense primer was 5'-GAAAATCCTCAATGAGGAAGAA-3', while several bases of the sense primer 5'-GGGATCCACCATGGTGTTGATGCC3' were mutated (underlined) to generate BamHI and Ncol restriction sites. Nucleotides 130-1662 of HB'-7 (B'-1) was excised and inserted into the PFI/APl site of pCR1. Since clone HB'-5 (B'-2) lacked a portion of the 5'-coding sequence, an AvrII/Apal fragment from this cDNA was ligated into the same site of pCR1-A to generate full-length pCR1-B'-2. HindIII and HincII fragments from pCR1-B'-1, containing the complete open reading frame, were subcloned into a mammalian expression vector, pRCMV (Invitrogen). An Hpal/Apal fragment was removed from pRCMV-B' and replaced with the corresponding fragment from pCR1-B'-2 to create pRCMV-B'-2. Donor mRNAs for cell expression were constructed by inserting BamHI/Nhel fragments from pRCMV-B'-2 and pRCMV-B'-1 into the BamHI/SnaI site of pBluescript KS (Stratagene). Sense and antisense primers of pRCMV-B'-1 and pRCMV-B'-2 were also inserted into pMammNeo (Clontech). B'-Halo/Sall fragments from the pMammNeo constructs were then subcloned into the BglII/Sall site of pFLAG-CMV2 (Eastman Kodak Co.) to generate plasmids for expression of epitope-tagged B'-1. The cDNA for the human B'-2 subunit of PP2A (13) was subcloned into the BamHI site of pFLAG-CMV2.

In Vitro Translation of B'- and GST-A Binding Assays—A PCR product was amplified from the mouse T-lymphocyte pACT-B'-3 plasmids using a sense primer 5'-TAATACGACTCACTATAGGGAGACCAACCATGGATGATGTATATAACTATCATTTC-3' and a random primer (5'-TAATACGACTCACTATAGGGAGACCA-3') was amplified by PCR from the mouse B'-3 PCR fragment, pRCMV-B'-1, and pRCMV-B'-2 as templates using the TnT T7 coupled reticulocyte lysate system (Promega). GST and GST-A-subunit fusion proteins were prepared from Escherichia coli and used in binding assays with the (15S)methionine-labeled B'-1 and B'-2 proteins (2). In some cases, purified bovine cardiac C subunit (2.5 µg) was preincubated for 30 min (4°C) with GST (16 µg) and the GST-A subunit fusion protein (16 µg) prior to addition of labeled B' translation products. Bound proteins were eluted with 10 mM glutathione, resolved by SDS-PAGE, followed by fluorography.

Association of Expressed B'- with the A and C Subunits of PP2A in Sf9 Cells—Recombinant baculoviruses encoding the B'- subunits were prepared in a baculovirus expression system (2) using the manufacturer’s protocol (Life Technologies, Inc.). Insect cells (4 x 10^7) were infected with recombinant B' baculoviruses (20), harvested 48 h postinfection, and washed twice with phosphate-buffered saline (PBS). Subsequent operations were performed at 4°C. The cells were resuspended in 3 ml of HS buffer (10 mM imidazole, pH 6.5, 1 mM EDTA, 1 mM diethyithorretol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol) and homogenized in a Dual homogenizer. The homogenate was centrifuged at 12,000 x g for 15 min, and the supernatant was loaded onto a heparin-Sepharose column (5 ml) equilibrated in HS buffer at a flow rate of 1 ml/min. The column was washed with the same buffer up to the A280 returned to base line. The column was eluted with 20 ml each of 0.1, 0.3, and 0.5 x NaCl in HS buffer, and 1 ml fractions were collected. A aliquots (20 µl) of the fractions were analyzed by immunoblotting with the B'-specific antiserum. An aliquot (0.25 ml) of fractions 40 (0.3 x NaCl elution) and 60 (0.5 x NaCl elution), which contained B'-, was adjusted to 0.5 ml with HS buffer and applied to a Superdex 200 10/30 gel filtration column in HS buffer containing 0.5 x NaCl. A second aliquot of fraction 60 was incubated with the purified AC form of bovine cardiac PP2A (20 µg) for 30 min prior to chromatography. Chromatography was carried out at a flow rate of 0.25 ml/min, and 0.5-ml fractions were collected. The fractions were analyzed by immunoblotting with antibodies against the C (24) and B' subunits.

Expression of B' in Mammalian Cells—Mouse NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Cells were transfected with pFLAG-B'-1 or pFLAG-B'-2, and transfected cultures were transfected using the liposome method (2). The cells were harvested 48 h posttransfection and washed twice with PBS. Cells were Dounce homogenized, and nuclei were isolated by centrifugation (800 x g, 10 min) through a 1 ml imidazole, pH 7.4, 0.25 M sucrose cushion. The supernatant was recentrifuged at 12,000 x g (10 min), and the resulting nuclear fraction was the cytosolic fraction. Nuclear and cytosolic extracts were prepared according to Krainer et al. (25). Cytosolic and nuclear proteins were resolved by SDS-PAGE and analyzed for the expression of B'-1, B'-2, and B' by immunoblotting.

Immunofluorescence Microscopy—NIH 3T3 cells transfected with pFLAG-B'-1, pFLAG-B'-2, or pFLAG-B'-2 were tpyrazinized after 24 h and grown on glass coverslips. The cells were fixed in 4% paraformaldehyde in ice for 5 min at −20°C and washed twice with PBS. Nonspecific sites were blocked by incubation in PBS containing 5% goat serum (1 h). Subsequent antibody incubations (1 h)
and washes were carried out in PBS containing 1% goat serum. The cells were incubated with monoclonal anti-FLAG 5 antibody (Kodak) at a concentration of 10 μg/ml followed by a Cy3-conjugated affinity-purified goat anti-mouse antibody (Jackson Immunoresearch Labs) at a dilution of 1:400. The coverslips were mounted with Fluoromount-G (Fisher) and examined with an Olympus AX70 microscope (600× magnification).

Production of B′α—Specific AntiserA—A synthetic peptide (CPQAQK-DPPKKDR) corresponding to amino acids 431–441 of B′α2 and residues 471–480 of B′α1 was coupled to keyhole limpet hemocyanin. The amino-terminal cysteine was added to facilitate the coupling. Peptide conjugation and polyclonal antibody production were carried out as described previously (13).

Protein Analyses—Protein concentrations were determined by the Bradford method (26) using bovine serum albumin as standard. SDS-PAGE was carried out in 0.75-mm-thick slab gels containing 7.5 or 10% acrylamide concentration (27). Immunoblot analyses were performed using Fab9 fragments of horseradish peroxidase-conjugated donkey anti-rabbit (1:10,000) or sheep anti-mouse (1:10,000) and the enhanced chemiluminescence system (Amersham Corp.) to detect bound antibody (13).

RESULTS

Molecular Cloning of cDNAs Encoding B′α—The major form of regulatory subunit in purified bovine cardiac PP2A is related to the B′ subunit originally identified in a form of rabbit muscle PP2A termed PP2Aα0 (17). Partial amino acid sequence of the bovine cardiac PP2A B′ subunit was obtained from tryptic and cyanogen bromide peptides. 15 peptides were sequenced, and a total of 177 residues were determined. A search of the protein data bases with these peptides indicated a high degree of similarity with the translated sequence of a randomly cloned human cDNA from myeloid KG1 cells (accession number D26445). The KG1 cDNA was 3.7 kb, and the longest open reading frame encoded for a protein of 475 amino acids. The deduced sequence of the KG1 cDNA contained 12 of the 15 peptides obtained from bovine cardiac B′. The similarity strongly suggested that the human cDNA was related to the bovine B′ subunit. A Saccharomyces cerevisiae cDNA, RTS1 (accession number U06630) encoding a suppressor of a defect in the ROX3 transcription factor was also found to contain some of the bovine B′ peptides. Although the similarity is much lower, it appears that this clone is a yeast homolog of the mammalian B′ subunit (Fig. 1).

Oligonucleotide primers were designed from the human sequence in the data base, and a 854-bp PCR product was amplified from a HUVEC λ ZAP cDNA library. The PCR product was sequenced, and was found to be identical to nucleotides 775–1629 of the KG1 cDNA. Five positive clones were isolated from the HUVEC cDNA library with the 854-bp probe using stringent hybridization and wash conditions. The largest cDNA (HB′-7) was 4064 bp long and nearly identical in sequence to the KG1 cDNA. Two base changes were present in the 3′ untranslated region of the HUVEC cDNA; nucleotide 2082 was G instead of A, and nucleotide 3847 was A instead of G. The HUVEC cDNA extends the 5′- and 3′-untranslated regions of the KG1 sequence by 62 and 182 nucleotides, respectively. The sequence around the putative initiation codon, AAGCAGATG-GTGG, conforms to the consensus motif for translation initiation in vertebrates (28). Four potential polyadenylation sites (AATAAA) are present at nucleotides 2480, 3677, 3851, and 4027. There is also an insertion of 117 nucleotides in the HB′-7 cDNA that is absent in the KG1 cDNA. The insertion occurs after nucleotide 1385 and encodes for an additional 39 amino acids (433–472). We have designated this cDNA B′α1. The deduced amino acid sequence encodes for a protein of 514 residues with a predicted molecular weight of 59,995 and an isoelectric point of 6.1 (Fig. 1).

The remaining cDNAs were found to be partial clones, three of which corresponded to B′α1. The fourth cDNA (HB′-5) lacked 617 nucleotides of the 5′ sequence, terminated at nucleotide 3138 of B′α1, and did not contain the 117-nucleotide insertion. This cDNA corresponded to the KG1 sequence in the database, and we have designated it B′α2. The deduced amino acid sequence encodes for a protein of 475 residues with a predicted molecular weight of 55,958 and an isoelectric point of 6.5 (Fig. 1).

An additional B′α cDNA was isolated in an independent screen of a mouse T-lymphocyte library for proteins that interact with the A subunit of PP2A using the yeast two-hybrid assay. One of the cDNAs that interacted with the GAL4 DNA binding domain-A subunit fusion protein was a mouse homolog of human B′α. This cDNA (B′α3) was 1.35 kb, lacked a portion of the 5′ sequence, and contained 37 nucleotides of the 3′ untranslated sequence. The partial open reading frame encodes for a protein of 435 residues, with a predicted molecular weight of 51,158. An alignment of the human, mouse, and a portion of the yeast RTS1 primary sequences is shown in Fig. 1. There are three conserved substitutions in the mouse sequence compared with human B′α1 (88% identity) and B′α2 (92% identity). The mouse sequence terminates 10 residues after the B′α1 insertion begins.

Based on the nucleotide sequence identity, the two human forms are likely to arise by alternative splicing of a single gene. A putative splice acceptor site boundary, CCCAGG (nucleotides 764–769 in B′α2, 1319–1324 in the KG1 cDNA, and 1379–1384 in B′α1), is present at the point of divergence between the B′α1 and B′α2 sequences. Consistent with these observations is the conservation of the same splice site junction in the mouse B′α3 cDNA (nucleotides 1231–1236). The presence of the 39-amino acid insertion in B′α1 produces a bipartite nuclear targeting signal (29) that is absent in both B′α2 and B′α3. The yeast homolog of B′α diverges from its mammalian counterparts at the amino and carboxyl termini, which are not shown in Fig. 1. The overall identity between the yeast protein and B′α1, B′α2, and mouse B′α3 is 39, 40, and 43%, respectively. However, if the divergent termini are omitted and the comparison is made on residues 127–561 of the yeast protein, the identity increases to 68%.

Expression and Distribution of B′α mRNA—The levels of transcripts encoding human B′α were analyzed in poly(A)+ RNA isolated from human tissues (Fig. 2A). A single major transcript of 4.4 kb was detected in all tissues examined. The B′α mRNA was very abundant in heart, skeletal muscle, and brain. Lower levels were present in the pancreas, kidney, lung, and placenta. Liver had a very low level of the B′α mRNA that was detectable with longer exposure times (data not shown).

The probe used in the Northern analysis does not distinguish between the alternatively spliced forms of human B′α. Therefore, expression of the alternatively spliced forms of B′α mRNA was examined in mouse tissues by reverse transcription PCR (Fig. 2B). Two major bands were amplified from each tissue that had mobilities identical to the B′α1 (717 bp) and B′α2 (600 bp) controls. Similar amounts of cDNA were present in each PCR reaction, since the ubiquitously expressed mRNA for cytoplasm was amplified to similar levels with all of the cDNAs (data not shown). Approximately the same ratios of B′α1 and B′α2 were observed in mouse tissues except liver and especially brain, where B′α2 was more prevalent. In contrast to the human Northern blot, mouse skeletal muscle contained significantly lower levels of the B′α transcripts. Interestingly, bands of ~650 and ~750 bp were also amplified with the heart, brain, and skeletal muscle cDNAs. Although we have not identified these products, they may be due to the presence of additional splice variants of B′α or other isoforms of the B′ regulatory subunit.

Association of Recombinant B′α Subunits with PP2A—Sev-
eral approaches were taken to show that the human and mouse B'α1, B'α2, mouse B'α3, and residues 127-561 of yeast RTS1. Peptides derived from purified bovine cardiac B' are shown below the aligned sequences. The asterisks represent identities, and the dashed lines indicate gaps. The bipartite nuclear localization signal in B'α1 is underlined. The nucleotide sequences of human B'α1 (accession number U37352), and mouse B'α3 (accession number U37353) have been deposited in the GenBank database. The accession number for human B'α2 (KG1 ORFY) is D26445 and for yeast RTS1 is U06630.

In vitro transcription/translation of B'α1 cDNAs in reticulocyte lysates directed the synthesis of [35S]methionine-labeled human and mouse B'α proteins. The expressed proteins had apparent molecular masses of 60 kDa (Fig. 3, lane 1, B'α1), 56-kDa (Fig. 3A, lane 2, B'α2), and 52-kDa (Fig. 3A, lane 3, B'α3), which were nearly identical to the predicted molecular weights of each splice variant. The interaction of the expressed proteins with the A subunit was assayed using a GST-A subunit fusion protein. A low level of binding to GST-A was observed with B'α1 (Fig. 3B, lane 1), B'α2 (Fig. 3C, lane 1), and B'α3 (Fig. 3D, lane 1). However, preincubation of the GST-A fusion protein with the C subunit of PP2A significantly enhanced the binding of all three forms of B'α (lane 2). The interaction of the B'α proteins with the A subunit was specific, as no binding was observed with GST alone (lane 3) or when GST was preincubated with the C subunit (lane 4). The minor bands present in the GST-A lanes may be due to proteolysis of B'α1 and B'α2 or initiation from an internal methionine.

Recombinant B'α proteins were also expressed in the baculovirus-insect cell system. Homogenates from infected cells were partially purified by heparin-Sepharose and size exclusion chromatography to determine if the expressed B'α proteins interacted with the endogenous AC core enzyme. The B'α proteins in infected SF9 cell extracts were eluted from heparin-Sepharose with both 0.3 and 0.5 M NaCl (data not shown). When B'α1 in the 0.3 M NaCl fraction was applied to a gel
fluorescence of NIH3T3 cells expressing FLAG-Bα correspond to the elution position of purified cardiac ACζ complexes was not disrupted by chromatography in 0.5 M NaCl. The 0.5 M NaCl heparin-Sepharose fraction, which eluted in fractions 27–29, corresponding to the predicted molecular weight of monomeric Bα (156,000). The 0.5 M NaCl filtration column, the peak of immunoreactivity co-eluted with a Bα specific probe as described under “Materials and Methods.” Panel B, first strand cDNA was prepared from poly(A+) RNA isolated from mouse tissues and used in a PCR with Bα specific primers as described under “Materials and Methods.” An aliquot of each reaction was resolved on a 1% agarose gel and stained with ethidium bromide (0.5 μg/ml). The migration of molecular size standards (kb) are indicated to the left of each panel.

**Table I**

| Transforming Plasmid | Recipient Strain |
|----------------------|------------------|
|                     | GAD-A | GAD-C |
| GDB-Bα1             | ++    | –     |
| GDB-Bα2             | +     | –     |
| GDB-p53             | –     | –     |
| GDB-A               | –     | +     |

**DISCUSSION**

We have isolated three novel cDNAs related to the bovine cardiac B′ regulatory subunit of PP2A. All three forms appear to be generated from a single gene by alternative splicing. Transcripts of B′α are widely expressed in human and mouse tissues and are especially abundant in muscle. The high level of B′α transcripts detected in heart and skeletal muscle is consistent with the biochemical composition of PP2A purified from these tissues (17, 24). These B′ subunits have no apparent homology to the B or PR72/130 (B′) regulatory subunits or other proteins that interact with and regulate PP2A, including viral tumor antigens and the phosphotyrosyl phosphatase activator protein (31). Interaction of these proteins with PP2A is not consistent with the biochemical composition of PP2A obtained from these tissues (17, 24). These B′ subunits have no apparent homology to the B or PR72/130 (B′) regulatory subunits or other proteins that interact with and regulate PP2A, including viral tumor antigens and the phosphotyrosyl phosphatase activator protein (31). Interaction of these proteins with PP2A is not consistent with the biochemical composition of PP2A obtained from these tissues (17, 24). These B′ subunits have no apparent homology to the B or PR72/130 (B′) regulatory subunits or other proteins that interact with and regulate PP2A, including viral tumor antigens and the phosphotyrosyl phosphatase activator protein (31). Interaction of these proteins with PP2A is not consistent with the biochemical composition of PP2A obtained from these tissues (17, 24). These B′ subunits have no apparent homology to the B or PR72/130 (B′) regulatory subunits or other proteins that interact with and regulate PP2A, including viral tumor antigens and the phosphotyrosyl phosphatase activator protein (31). Interaction of these proteins with PP2A is not consistent with the biochemical composition of PP2A obtained from these tissues (17, 24).

**Fig. 3. Binding of B′α proteins to a GST-A subunit fusion protein.** Panel A, SDS-PAGE of in vitro translation products of human B′α (lane 1), human B′α2 (lane 2), and mouse B′α3 (lane 3) cDNAs. A control sample containing no cDNA template was applied to lane 4. An aliquot of the translation mixture containing B′α1 (panel B), B′α2 (panel C), and B′α3 (panel D) were assayed for binding to a GST-A subunit fusion protein as described under “Materials and Methods.” Lane 1, GST-A subunit; lane 2, GST-A subunit + + purified bovine C subunit (2.5 μg); lane 3, GST fusion protein; lane 4, GST fusion protein + + purified bovine C subunit (2.5 μg). The migration of molecular mass standards including bovine serum albumin (66-kDa), glutatione dehydrogenase (55-kDa), ovalbumin (44-kDa), and aldolase (40-kDa) are shown to the left of each panel.

staining in contrast, transient expression of FLAG-Bα (Fig. 5E) led only to cytoplasmic staining. The predominantly cytoplasmic localization of FLAG-Bα is consistent with the distribution of endogenous Bα in CV1 cells (30). Similar amounts of FLAG-Bα1 and FLAG-Bα2 were present in whole cell extracts as determined by immunoblotting with the anti-FLAG antibody (data not shown). The differential localization of FLAG-B′α1 and FLAG-B′α2 in the nuclei and cytoplasm and FLAG-Bα in the cytoplasm was confirmed by immunoblots of cytosolic and nuclear extracts from the transfected cells (data not shown). Control experiments in which the primary or secondary antibody was omitted led to a loss of immunostaining. Preadsorption of the anti-FLAG antibody with the antigenic peptide also abolished the immunoreactivity (data not shown). Phase contrast images (Fig. 5, B, D, and F) showed that non-transfected cells lacked any significant immunofluorescence.

Localization of B′α in Mammalian Cells—Indirect immunofluorescence of NIH3T3 cells expressing FLAG-B′α revealed both cytoplasmic and nuclear populations of the recombinant proteins. Transient expression of both B′α1 (Fig. 5A) and B′α2 (Fig. 5C) led to diffuse cytoplasmic and pronounced nuclear staining. In contrast, transient expression of FLAG-Bα (Fig. 5E) led only to cytoplasmic staining. The predominantly cytoplasmic localization of FLAG-Bα is consistent with the distribution of endogenous Bα in CV1 cells (30). Similar amounts of FLAG-B′α1 and FLAG-B′α2 were present in whole cell extracts as determined by immunoblotting with the anti-FLAG antibody (data not shown). The differential localization of FLAG-B′α1 and FLAG-B′α2 in the nuclei and cytoplasm and FLAG-Bα in the cytoplasm was confirmed by immunoblots of cytosolic and nuclear extracts from the transfected cells (data not shown). Control experiments in which the primary or secondary antibody was omitted led to a loss of immunostaining. Preadsorption of the anti-FLAG antibody with the antigenic peptide also abolished the immunoreactivity (data not shown). Phase contrast images (Fig. 5, B, D, and F) showed that non-transfected cells lacked any significant immunofluorescence.
GAL4 activation domain-A subunit fusion protein in the yeast two-hybrid assay. A role for the C subunit in stabilizing oligomeric complexes was originally suggested by reconstitution experiments with porcine cardiac PP2A (32). This idea is supported by chemical cross-linking studies (13, 20), and analysis of A subunit mutants (33, 34). The increased binding of recombinant B'α proteins to GST-A in the presence of C is also consistent with a role in stabilizing the AC:B' heterotrimer. We do not know if the endogenous yeast A subunit participates in the two-hybrid interaction. However, no hybrids were formed between GAD-C and GDB-B'α, suggesting that B'α has very weak affinity for C and that the yeast A subunit homolog does not participate in hybrid formation. Another possibility is that the fusion with the DNA binding domain to the amino terminus of B'α hinders interaction with GAD-C.

The interaction assays suggested that the B'α subunits have different apparent affinities for PP2A. Of the three proteins, B'α1 displayed the strongest interaction in both the yeast two-hybrid and GST-A binding assays. It is not known whether the absence of 8 amino acids from the amino terminus of mouse B'α2 (panels B and D) were partially purified on a heparin-Sepharose column. Aliquots of the fraction eluted with 0.3 M NaCl (panels A and B) and 0.5 M NaCl (left half of panels C and D) were separately chromato- graphed on a Superdex 200 10/30 column. A second aliquot of the 0.5 M NaCl fractions (right half of panels C and D) were incubated with purified bovine AC (20 µg) for 30 min (4°C) prior to chromatography. The fractions from the Superdex column were analyzed by immunoblotting for B'α1 (panels A and C), B'α2 (panels B and D), and the C subunit of PP2A (panels A and B). The homogenates were used as positive controls (+) for the expression of B'α1 and B'α2. The migration of molecular mass standards including bovine serum albumin (66-kDa), glutamate dehydrogenase (55-kDa), and aldolase (40-kDa) are shown to the left of each panel.

FIG. 4. In vivo and in vitro reconstitution of expressed B'α proteins with the AC core enzyme of PP2A. Homogenates of insect cells infected with baculoviruses encoding B'α1 (panels A and C) and B'α2 (panels B and D) were partially purified on a heparin-Sepharose column. Aliquots of the fraction eluted with 0.3 M NaCl (panels A and B) and 0.5 M NaCl (left half of panels C and D) were separately chromato- graphed on a Superdex 200 10/30 column. A second aliquot of the 0.5 M NaCl fractions (right half of panels C and D) were incubated with purified bovine AC (20 µg) for 30 min (4°C) prior to chromatography. The fractions from the Superdex column were analyzed by immunoblotting for B'α1 (panels A and C), B'α2 (panels B and D), and the C subunit of PP2A (panels A and B). The homogenates were used as positive controls (+) for the expression of B'α1 and B'α2. The migration of molecular mass standards including bovine serum albumin (66-kDa), glutamate dehydrogenase (55-kDa), and aldolase (40-kDa) are shown to the left of each panel.

PP2A has generally been regarded as a soluble cytoplasmic enzyme, but significant amounts of PP2A are also present in the nucleus (35, 36). Partial purification of rat liver nuclear PP2A suggests that the nuclear enzyme is a heterotrimer; however, the type of B subunit in nuclear PP2A has not been identified (37). A significant feature of all three B'α variants is the presence of a cluster of basic residues near the carboxyl terminus of B'α3 affects binding to PP2A. However, there is evidence that a portion of the amino terminus of the B'α and Bβ subunits is involved in binding to the AC core enzyme (13). The fact that B'α1 and B'α2 have different apparent affinities for the AC complex suggests that regions near the carboxyl terminus are also important for subunit interactions.

The purified bovine cardiac B' subunit has a very high affinity (IC50 = 0.58 nM) for the AC form of PP2A (20). Although cardiac B' α and B'α2 have the same mobility in SDS-PAGE, functional data and other lines of evidence suggest that human B'α2 and bovine B' are not the same protein. Three of the peptides derived from the bovine cardiac protein were not found in the human or mouse B'α sequences. Although these peptides may have been generated from a contaminant(s), they were not similar to any other sequences in the data bases. Anti-peptide antibodies raised against the human B'α proteins cross-reacted with the bovine cardiac subunit; however, no cross-reactivity with the human proteins was observed with an antiserum (E005) raised against purified bovine cardiac B' (data not shown). Taken together, these data indicate that the human B'α and bovine cardiac B' subunits are closely related isoforms of a larger B' family.

Two While this manuscript was under review, McCright and Virshup (39) reported the isolation of a new family of PP2A regulatory subunit (accession numbers L42373–L42375). Comparison of the sequences indicates that their cDNAs are members of the B' family of regulatory subunits. The B56y isoform that they report corresponds to our B'α cDNAs.
terminus and a consensus bipartite nuclear localization signal in B′α1 (residues 462–478). The bipartite motif has been found in 60% of all known nuclear proteins and less than 4% of nonnuclear proteins (29). Although the bipartite nuclear localization signal is absent in B′α2, localization of FLAG-B′α2 was similar to that of B′α1 in our transient assays. While some nuclear proteins contain large T antigen-type nuclear targeting signals or the bipartite motifs, there are many nuclear proteins that do not contain a consensus sequence for nuclear localization (29). The only significant feature that has been recognized in these proteins is the presence of clusters of basic residues within the signaling domain. The basic residues present in the carboxyl termini of the B′α proteins may serve as a signal for nuclear import. Mutational analysis of this region will be required to determine the critical residues for nuclear localization.

Exclusion of transiently expressed FLAG-B′α from the nucleus is consistent with previous immunofluorescence data showing that AC-B′α is largely cytoplasmic and that a subpopulation is associated with the microtubule cytoskeletal network (30). We have not quantitated the amount of FLAG-B′α that is associated with endogenous AC in the transient assays. However, other studies have shown that expressed small T antigen interacts with endogenous AC in mammalian cells (2, 38). In addition, we have shown that expressed B′ α subunits form heterotrimeric complexes with endogenous AC in S9 cells. Presumably, some FLAG-B′α associates with AC and is bound to microtubules, preventing nuclear import. We have no evidence that AC-B′α binds to microtubules, and failure of the AC-B′α complex to interact with the cytoskeleton may allow nuclear uptake. Regardless of the mechanism, it is clear that expressed B′α subunits are highly localized within the nucleus, while the B′α subunit is not. This result suggests that some members of the B′ family may be present in nuclear PP2A and that B′ may be important in nuclear functions of PP2A. PP2A has been implicated in the dephosphorylation of nuclear cAMP-response element binding protein (37, 38). The AC-B′α heterotrimer may be involved in the control of cAMP-mediated changes in gene transcription. Yeast RTS1 is a suppressor of a mutation in the ROX3 transcription factor. The similarity of RTS1 and B′ is also significant, and the mammalian homolog in regulating the activity of transcription factor(s).

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