Identification of a New Family of Protein Phosphatase 2A Regulatory Subunits*

(Received for publication, June 14, 1995, and in revised form, August 11, 1995)

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Protein phosphatase 2A (PP2A) is a major intracellular protein phosphatase that regulates multiple aspects of cell growth and metabolism. The ability of this widely distributed heterotrimeric enzyme to act on a diverse array of substrates is largely controlled by the nature of its regulatory B subunit. Only two gene families encoding endogenous B subunits have been cloned to date, although the existence of several additional regulatory subunits is likely. We have identified by two-hybrid interaction a new human gene family encoding PP2A B subunits. This family, denoted B56, contains three distinct genes, one of which is differentially spliced. B56 polypeptides co-immunoprecipitate with PP2A A and C subunits and with an okadaic acid-inhibitable, heparin-stimulated phosphatase activity. The three B56 family members are 70% identical to each other but share no obvious homology with previously identified B subunits. These phosphatase regulators are differentially expressed, with B56α and B56β highly expressed in heart and skeletal muscle and B56δ highly expressed in brain. The identification of this novel phosphatase regulator gene family will facilitate future studies on the control of protein dephosphorylation and the role of PP2A in cellular function.

Protein phosphatase 2A (PP2A) is a major intracellular phosphatase that regulates such diverse cellular processes as DNA replication, transcription, signal transduction, and intermediary metabolism (1–3). PP2A is a heterotrimer, containing A, B, and C subunits. The catalytic activity of PP2A resides in the C subunit, a 36-kDa protein encoded by two 97% identical genes. The C subunit binds stably to the carboxyl-terminal region of the A subunit, a 65-kDa rod-shaped polypeptide consisting of 15 imperfect repeats. The B subunits bind to the amino-terminal region of the A subunit (Fig. 1A) and determine the substrate specificity of the complex (4–8). Three distinct B subunits have been biochemically isolated from a variety of mammalian tissues (9–14), and several studies have suggested the existence of additional B subunits (15, 16). Additionally, several DNA tumor viruses encode polypeptides that can function as PP2A B subunits (17–19). The B subunits purified to date migrate in SDS-PAGE with the apparent molecular masses of 54 kDa (B54), 55 kDa (B55), and 72 kDa (B72). Three cDNAs encoding 55-kDa B subunits have been identified (10, 11, 20); the B55 family members are 80–90% identical, and their level of expression varies by tissue type. A cDNA encoding the 72-kDa B subunit has also been cloned, and a splice variant encoding a 130-kDa protein has been identified (12). The sequence of the 54-kDa B subunit cDNA has not yet been reported. Interestingly, the amino acid sequences of the B55 and B72 subunits and the viral PP2A binding proteins show little homology to each other; thus, no common motif mediating the interaction of the B subunit with the PP2A A and C subunits has been discovered.

Heterotrimeric PP2A enzymes with different B subunits have distinct substrate specificities (7, 8, 21), a mode of phosphatase regulation that has important functional effects. For example, PP2A can turn SV40 DNA replication on or off, depending on the type of B subunit in the holoenzyme (6, 22). Viral replication in vitro is controlled by the activity of viral initiator phosphoprotein, SV40 large T antigen. The heterotrimeric form of PP2A containing B55 removes a phosphoryl group from threonine 124 (a cyclin-dependent kinase site) (23) and inactivates T antigen's ability to initiate SV40 DNA replication, while the PP2A heterotrimer containing B72 removes inhibitory phosphoryl groups from serines 120 and 123 (casein kinase I sites) (24, 25) and activates SV40 DNA replication (6).

An additional role of B subunits may be to act as targeting subunits that direct PP2A to specific subcellular locations. This method of regulation has been most clearly demonstrated for protein phosphatase 1, where the catalytic subunit is localized to its substrates, e.g. phosphorylase, phosphorylase kinase, and glycogen synthase, by association with a specific glycogen-binding subunit (2). Similarly, PP2A-B55α has recently been shown to associate with microtubules (26). While PP2A activity has also been found in membrane and nuclear fractions, the B subunits of PP2A that direct the heterotrimer to these sites have not yet been identified.

This current study was designed to identify novel PP2A B subunits. Using the yeast two-hybrid method (27) with the PP2A A subunit as bait, we identified a novel gene family encoding three polypeptides with a predicted size of approximately 56 kDa that were 70% identical to each other but with no significant similarity to either B55 or B72. Full-length polypeptides expressed in 293 cells bound to PP2A A and C subunits and co-immunoprecipitated with a heparin-stimulated, okadaic acid-inhibited phosphorylase phosphatase activity. Northern blots of human tissue showed that these genes...
have tissue-specific expression patterns, with two isoforms highly expressed in heart and skeletal muscle and one highly expressed in the brain. These results are further evidence that protein serine/threonine phosphatase diversity is generated in large part by association of a common catalytic subunit with an highly expressed in heart and skeletal muscle and one highly expressed in the brain. These results are further evidence that protein serine/threonine phosphatase diversity is generated in large part by association of a common catalytic subunit with a highly expressed in heart and skeletal muscle and one highly expressed in the brain. These results are further evidence that protein serine/threonine phosphatase diversity is generated in large part by association of a common catalytic subunit with a highly expressed in heart and skeletal muscle and one highly expressed in the brain. These results are further evidence that protein serine/threonine phosphatase diversity is generated in large part by association of a common catalytic subunit with a highly expressed in heart and skeletal muscle and one highly expressed in the brain.

**EXPERIMENTAL PROCEDURES**

Yeast Two-hybrid Screen Strains and Constructs—A two-hybrid screen based on the method of Fields and Song (28) (Fig. 1) was performed using the Saccharomyces cerevisiae strain L40 (MATa, his3Δ1200, trp1–901, leu2–3, 112, ade2, lys2, URA3 (LexA-op) lacZ, ura3–52, trp1–901, leu2, ADH1 (LexA-op) lacZ (constructed by S. Hollenberg). AM70 (MATa, his3, leu2, trp1–901, ade2, URA3::(lexAop), His3, Trp1, Leu2, URA3::(lexAop)) contains inserts averaging 1500 bp in length and forms a control strain for background specificity. In pilot experiments, plasmids were cloned by use of polymerase chain reaction using PCR primers (Table I) added appropriate restriction sites for in-frame insertion into the Yeast two-hybrid method (Fig. 1) (32, 34).

The bait plasmid was based on pBT116 (originally constructed by P. Bartel and S. Fields) with the selectable marker TRP1 and modified by the insertion of the ADE2 gene (31) in the p411 site) and expresses LeA fused to the full-length human PP2A Aα subunit (5, 18). Expression of this construct, LexA-65A, was confirmed by immunoprecipitation of the fusion protein from [35S]methionine-labeled yeast extracts with an antibody to the PP2A Aα subunit. Constructs encoding mutant A subunits were also expressed in S. cerevisiae strain L40 and were identified by accumulation of a red metabolite due to loss of the ADE2 gene also carried on the bait plasmid. Yeast containing only the library plasmids encoding putative interacting proteins were then tested for specific interaction by mating to AM70 yeast carrying plasmids encoding LexA-65A, Lexa-SUB, Lexa-A315, Lexa-397, or Lexa-A-lamin (Fig. 1) (32, 34).

Full-length clones were isolated from a human fetal brain cDNA library in bacteriophage lambda by standard methods (35) and sequenced on both strands using Sequenase 2.0 according to the manufacturer's instructions.

Expression of Hemagglutinin (HA)-tagged B Subunits—The protein coding sequences of B55α (20), B56α, and B56β were cloned using the indicated polymerase chain reaction primers into pCEP-4/LENCer (a CMV promoter-driven expression vector based on pCEP4 (Invitrogen) that encodes a hemagglutinin epitope tag (36) at the amino terminus of the expressed polypeptide). 293 cells (human embryonic kidney cells transformed with adenovirus) grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) plus 10% supplemented calf serum were transfected using lipofectamine (Life Sciences) according to the manufacturer's instructions. Using a plasmid that expresses lacZ from the CMV promoter, the transfection efficiency was determined to be about 40%. Cells were lysed 24–36 h after transfection on ice in 20 ml Tris-HCl, pH 7.5, 0.2% Nonidet P-40, 10% glycerol, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 1 mM benzamidine, and 2 μM pepstatin. Lysates were then centrifuged at 14,000 × g for 3 min, and complexes were immobilized from the supernatant by the addition of 12C5 mAb (a mouse monoclonal antibody that recognizes the hemagglutinin epitope) (36) and protein A-agarose. For immunoblotting, washed immobilized precipitates were solubilized in 1 × SDS-PAGE loading buffer, separated by SDS-PAGE on 10% gels, transferred to nitrocellulose, then probed with rabbit anti-PP2A-A and PP2A-C antibodies and visualized by enhanced chemiluminescence (Amersham). Phosphatase activity in the immunoprecipitate was determined by quantitating the release of trichloroacetic acid-soluble [32P]phosphate from [32P]orthophosphate (37). All phosphatase assays were performed in duplicate, and the amount of PP2A activity was calculated by subtracting radioactivity released from immunoprecipitates of untransfected cells or cells transfected with a HA-ERK1 construct.

Northern Blot Analysis—A multiple tissue human Northern blot (Clontech) was probed sequentially with [32P]labeled cDNA encoding B56 (800-bp Ncol-EcoRI fragment), B56γ (605-bp EcoRI fragment), and B56β (entire prey plasmid isolated by the two-hybrid method) and human β-actin (supplied by Clontech).

Sequence analysis was performed with the Wisconsin package (38) and GenBank searches using the BLAST algorithm (39).
expressing the fusion proteins VP16-B55, GAL4-B72, and GAL4-C subunit were constructed and tested for interaction with LexA-65A. The GAL4-B72 fusion protein interacted strongly and specifically with LexA-65A (Fig. 1), demonstrating the feasibility of identifying PP2A B subunits with the two-hybrid method.

A HeLa cell cDNA library in pGAD-GH was then screened for proteins that interact with the LexA-65A bait (Fig. 1). 17 × 10^6 transformants were plated on His^- plates, and rapidly growing colonies were further screened by blue/white assay for lacZ expression and for specific interactions in a mating assay. 212 clones were isolated that interacted strongly and specifically with the A subunit. These 212 clones were separated into 16 groups based on dot blot hybridization and restriction digests of polymerase chain reaction-amplified inserts. The 5' and 3' ends of representative clones from each of the 16 groups were then sequenced. The three groups that are the subject of this report encoded polypeptides that are 70% identical to each other and are referred to as B56g, B56h, and B56j. Five clones encoded B56g, two clones encoded B56h, and one clone encoded B56j.

To identify regions of the PP2A subunit bait required for interaction with these putative B subunits, yeast strain AMR70 carrying mutant bait constructs (as described under "Experimental Procedures") were mated to L40 yeast carrying AMR70 carrying mutant bait constructs (as described under "Experimental Procedures") for interacting proteins. Additional LexA constructs (LexA-315, LexA-397, LexA-SUB, and LexA-lamin) were used to characterize the interaction between the PP2A A subunit and putative B subunits. Interaction between the LexA constructs and the GAL4 fusion proteins activate transcription of HIS3 and lacZ genes in S. cerevisiae L40 and AMR70 cells. B56 clones interact specifically and distinctively with the PP2A A subunit. L40 cells expressing the indicated B subunit fusion proteins were mated to AMR70 cells expressing LexA-65A, LexA-modified A subunits, or the nonspecific bait LexA-lamin. The resulting diploids were tested for ability to grow on His^- plates. + signifies growth was similar to growth on His^- plates, ++ indicates growth was less than half the rate of growth on His^- plates, and – indicates no growth. SV40 small t antigen (ag) was not tested in the mating assay; the data indicated here in italics are predicted from the results of Ruediger et al. (4, 5).

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**Fig. 1. Application of the two-hybrid method to identify PP2A B subunits.** A, subunit interactions in the PP2A heterotrimer and a schematic of the fusion proteins used. The PP2A B and C subunits are thought to bind to the amino and carboxyl regions of the A subunit as shown (4). The 65-kDa A subunit was expressed as a fusion with the LexA protein (LexA-65A). It was used to screen a HeLa cell cDNA library in pGAD GH (see "Experimental Procedures") for interacting proteins. Additional LexA constructs (LexA-315, LexA-397, LexA-SUB, and LexA-lamin) were used to characterize the interaction between the PP2A A subunit and putative B subunits. Interaction between the LexA constructs and the GAL4 fusion proteins activate transcription of HIS3 and lacZ genes in S. cerevisiae L40 and AMR70 cells. B56 clones interact specifically and distinctively with the PP2A A subunit. L40 cells expressing the indicated B subunit fusion proteins were mated to AMR70 cells expressing LexA-65A, LexA-modified A subunits, or the nonspecific bait LexA-lamin. The resulting diploids were tested for ability to grow on His^- plates. + signifies growth was similar to growth on His^- plates, ++ indicates growth was less than half the rate of growth on His^- plates, and – indicates no growth. SV40 small t antigen (ag) was not tested in the mating assay; the data indicated here in italics are predicted from the results of Ruediger et al. (4, 5).
and B56 

12CA5 mAb (Fig. 3) predict molecular weight, cytosolic extracts from transfected cells verified that these constructs indeed encoded proteins of the expected size. Soluble extracts from transfected cells were separated by SDS-PAGE on a 10% gel and then transferred to a nitrocellulose membrane. HA-tagged proteins were detected by immunoblotting with the 12CA5 mAb. Asterisk indicates a 12CA5 mAb cross-reacting band present in untransfected cells. B and C, soluble extracts from transfected cells were subjected to immunoprecipitation with 12CA5 mAb and protein A-agarose. Immunoprecipitates were eluted in 1× SDS-PAGE loading buffer, separated on a 10% polyacrylamide gel, and transferred to a nitrocellulose membrane, where they were probed with affinity-purified rabbit antibodies that recognize the PP2A A and C subunits. As a positive control, whole lysate was run in lane A (EXTRACT). Asterisk indicates IgG heavy chain present in the immunoprecipitates.

Finally, to evaluate whether the B56 polypeptides bound to PP2A heterotrimers (forming heterotetramers) or formed novel heterotrimers, soluble extracts from transfected cells were analyzed by glycerol gradient velocity sedimentation followed by immunoblot analysis with 12CA5 mAb. The majority (~75%) of HA-B56α migrated at about the same speed as HA-B55α and HA-B56β migrates at about the same speed as HA-B55β.

Having established that the constructs indeed produced soluble proteins of the expected size, extracts from transfected cells were subjected to immunoprecipitation with 12CA5 mAb. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with antibodies that recognize PP2A A and C subunits. As Fig. 3, B and C, demonstrates, HA-tagged B56α and B56β polypeptides co-immunoprecipitate with PP2A A and C subunits in transfected human cells, indicating that B56 forms a stable complex with PP2A in human cells.

PP2A Phosphatase Activity Co-immunoprecipitates with HAtagged B56α and B56β—PP2A heterotrimers contain a catalytic (C) subunit whose phosphatase activity is (i) inhibited by nanomolar concentrations of okadaic acid and (ii) stimulated by micromolar concentrations of okadaic acid strongly suggests that the phosphatase activity present in these assays is PP2A. Phosphorylase phosphatase phosphatase activity was also increased 2-4-fold in the presence of 15 μg/ml heparin (Fig. 4B), a result expected for PP2A complexes. Thus, the data strongly suggest that the B56 polypeptides are part of a stable and active PP2A complex.

Tissue-specific Expression of B56 Isoforms—The previously cloned PP2A regulatory subunits have shown tissue-specific and developmentally regulated patterns of gene expression, with specific isoforms preferentially expressed in muscle and brain (10, 13). To determine the expression pattern of the B56 genes, a multiple human tissue Northern blot was probed with sequences from the three B56 genes (Fig. 5). B56α mRNA is approximately 3500 nucleotides, with expression in all tissues examined but with the highest expression in heart and skeletal muscle. B56β mRNA is 3000 nucleotides with highest expression in brain. The apparent sizes of B56α and B56β mRNAs (3.5 and 3.0 kb) are 380–550 nucleotides longer than the cDNA clones we have obtained. B56α probe hybridizes with three mRNA species of 1.8, 2.1, and 4.4 kb. The 4.4-kb transcript is most highly expressed in heart and skeletal muscle, while the shorter transcripts are present largely in heart alone. The 4.4-kb species most likely corresponds to the 3700-bp HumORFY sequence, while the smaller more cardiac-specific mRNAs most likely correspond to the B56β1 clone isolated in this study.
We now report the identification, using the two-hybrid method, of a new gene family encoding PP2A regulatory subunits. These genes encode authentic PP2A B subunits based on the following evidence. First, all members of the gene family B56 (α, β, γ) interacted with a LexA-A subunit bait in the two-hybrid assay and failed to interact with irrelevant baits such as LexA-lamin. Second, PP2A A and C subunits were shown to associate with epitope-tagged B56α and β polypeptides in human cells in co-immunoprecipitation assays. Third, HA-B56α and β co-immunoprecipitated with a phosphatase activity that was inhibited by 1 nmol of okadaic acid and enhanced by heparin, the results expected for a component of a PP2A complex.

It is of note that the B56 gene family has no obvious similarity to previously identified gene families encoding polypeptides that bind to the amino-terminal end of the PP2A A subunit (B72/130, B55, B56, and polyoma and SV40 small t antigens). Thus, it has not been possible to define an interaction domain that is required for binding to the A subunit of PP2A. The B56 family has a very highly conserved (80% identical) central region, while both the carboxyl terminus and the amino terminus are significantly more divergent. This suggests that the conserved region is required for interaction with the A and possibly the C subunit, whereas the ends may perform different functions such as regulation of substrate specificity or intracellular location of PP2A.

The lack of sequence similarity between the different B subunit families suggests that they each bind differently to the PP2A core A-C complex and exert their effects on substrate specificity in this manner. In support of this hypothesis, we noted that test prey encoding B55, B72, and B56 fusion proteins each interacted differently in the two-hybrid screen when tested for association with the mutant and wild type A subunits (Fig. 1B). Thus, B72, unlike B55 and B56, interacted with carboxyl-truncated A subunit A397, and the B56 prey interacted with full-length A subunit significantly more strongly than did the B55 prey. B55 interaction with the A subunit is reportedly stabilized by the presence of the C subunit (4); this is supported by our finding that the B55 prey construct interacted only weakly with the A bait in yeast, where no mammalian C subunit exists. B72 and B56, like SV40 small t antigen, may bind more tightly to the A subunit and thus have less of a requirement for a B-C interaction. Additionally, within each B subunit family there are non-conserved sequences that may contribute to unique interactions with the PP2A A and C subunits or contribute to interactions with additional cellular proteins. For example, PP2A heterotrimers containing either B55α or B55β differ in their response to effector molecules such as proteamine and heparin (44). Differential expression in diverse tissues of B56 and B55 family members also implies that each isoform performs a specific function.

A yeast gene 68% similar to the B56 gene family has been independently identified by two groups (Fig. 2) (24, 25). SCS1 (suppressor of chaperonin sixty-1), was isolated as a high copy suppressor of several temperature-sensitive alleles of hsp60 (a mitochondrial chaperonin) (24). SCS1 in budding yeast is a cytosolic protein that when overexpressed appears to positively regulate transcription of additional chaperonin genes. The identical gene, termed RTS1, was cloned as a multicopy suppressor of several temperature-sensitive alleles of hsp70 (25). Interestingly, a role for both RT51 and SCS1 in the cytosolic response to anaerobic conditions has been suggested. One function of the B56 homologues in yeast appears to be as regulators of the transcriptional response to environmental stress.

The PP2A regulatory subunits we have identified are similar in size and tissue distribution to a previously purified regulator
of PP2A with an M, of 54,000 as assessed by SDS-PAGE (13). If the B56 genes indeed encodes the 54-kDa protein, we would predict based on Northern blot analysis that the cardiac B subunit, also known as B', is encoded by B56a or B56γ.

PP2A has been implicated in the control of the cell cycle and the initiation of DNA replication. Since the PP2A A and C subunits are distributed in multiple cellular compartments, one function of a B subunit may be to target the heterotrimer to the nucleus, where it can participate in the regulation of these processes. The expression of the B55, B56, and B72 genes largely in terminally differentiated tissues such as heart and brain suggests that the PP2A B subunit that has direct involvement in cell cycle regulation or DNA replication remains to be identified.

Acknowledgments—We thank Rolf Ruediger, Gernot Walter, David Pallas, Jozef Goris, and Brian Hemmings for plasmids encoding wild type and mutant PP2A subunits; Stan Hollenberg and Graeme Bolger for plasmids and yeast strains; Kimberly Fish for assistance with methods; and John Phillips, David Stillman, and Andrew Thorburn for helpful discussions.

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