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Cloning and characterization of Bδ, a novel regulatory subunit of protein phosphatase 2A

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Abstract Variable regulatory subunits of protein phosphatase 2A (PP2A) modulate activity, substrate selectivity and subcellular targeting of the enzyme. We have cloned a novel member of the B type regulatory subunit family, Bδ, which is most highly related to Bα. Bδ shares with Bα epitopes previously used to generate subunit-specific antibodies. Like Bα, but unlike Bβ and Bγ which are highly brain-enriched, Bδ mRNA and protein expression in tissues is widespread. Bδ is a cytosolic subunit of PP2A with a subcellular localization different from Bα and may therefore target a pool of PP2A holoenzymes to specific substrates.

Key words: Phosphoprotein phosphatase; Phosphorylation; Signal transduction; Protein phosphatase 2A; Regulatory subunit; Subcellular fraction

1. Introduction

Serine/threonine phosphatase activity in cells can largely be attributed to four major enzyme classes: type 1 (PP1), type 2A (PP2A), type 2B (PP2B or calcineurin), and type 2C (PP2C) (reviews: [1–5]). The catalytic subunits of PP1, PP2A, and PP2B are complexed with a variety of regulatory subunits, thought to impart differential catalytic properties and subcellular distribution.

PP2A is a heterotrimeric phosphatase, composed of A, B, and C subunits. The structural A and catalytic C subunits form the constitutive core of the enzyme, which associates with one of a large number of regulatory B subunits. Three families of B subunits have been identified thus far. The prototypical B (or PR55) family of subunits is encoded by three genes, Bα, Bβ [6], and Bγ [7,8]. The B′ (or B56) family consists of at least seven splice variants derived from five genes [9–13]. RNAs for the two members of the B″ family, PR72 and PR130, arise by alternative splicing or use of alternate transcription start sites from the same gene [14]. Primary structure as well as functions of B and B′ subunits are conserved from yeast to mammals [15].

Using a homology-based cloning approach, we report here the identification of a novel isoform of the B class of PP2A regulatory subunits. Bδ is widely expressed in rat tissues and appears to be involved in targeting PP2A to the cytosolic compartment.

2. Materials and methods

2.1. Cloning of Bδ

Homology-based reverse transcriptase-polymerase chain reaction (RT-PCR) cloning was used to isolate partial B subunit cDNAs from total adult rat brain RNA [16]. Briefly, a degenerate antisense oligonucleotide primer corresponding to a C-terminal region of amino acid homology in the known B subunits (ENIIAVA [Bα 429–435]) was used in the reverse transcription reaction to generate a mixture of B subunit cDNAs. Additional sets of degenerate oligonucleotide primers corresponding to conserved sequences (sense: NIVDIKP [Bα 213–219], FEC[ACV]WNG [Bα 346–353]; antisense: ENIIAVA [Bα 429–435], HTAWHP [Bα 421–426]) extended with restriction sites (sense: *Kpn*I, antisense: *Eco*R1) were used for PCR amplification of the cDNAs. Partial cDNAs were ligated into pBluescript II KS and sequenced. From a total of 94 clones, 29 were identified as Bα, 34 as Bβ, 27 as Bγ, and four corresponded to a novel sequence, which was termed Bδ. Additional 5′ sequence of Bδ was obtained by 5′ rapid amplification of cDNA ends (5′-RACE) from rat testis total RNA according to instructions supplied with the Marathon cDNA amplification kit (CLONTECH). The 1018 bp 5′-RACE product was blunt-end ligated into pBluescript II KS, and used to screen an adult rat brain cDNA library according to standard protocols. A 2034 bp clone was isolated, sequenced on both strands, and subcloned into pBluescript II KS and pcDNA3 (Invitrogen, Carlsbad, CA, USA), a mammalian expression vector under control of the cytomegalovirus promotor. The Bδ sequence has been deposited in GenBank under accession number AF180350.

2.2. Ribonuclease protection assays

Ribonuclease protection analyses were performed as described [16] using [^{32}P]-labeled riboprobes (236 or 650 bases) synthesized from partial B subunit cDNAs obtained by RT-PCR (see Section 2.1); a cyclophilin probe was used as an internal control for equal RNA loading. Total RNA was isolated by the cesium-chloride method [17], or with TriReagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's recommendations.

2.3. Antibody generation

A synthetic peptide corresponding to an N-terminal sequence of Bδ (GGGCPTGGNDFQW) was coupled to keyhole limpet hemocyanin, and used to immunize rabbits (Bionostics, Inc., Toronto, Canada). Antisera were affinity-purified as described [18]. Reliable detection of endogenous Bδ by immunoblotting required purification of phosphatases by microcystin-Sepharose (see Section 2.4), suggesting that Bδ is a low abundance subunit and/or that the antibody has low affinity (owing perhaps to the presence of several low antigenicity glycines in the immunogenic peptide). Antibodies to other PP2A subunits used in this study have been described before [19].

2.4. Microcystin-Sepharose affinity purification of PP2A from transfected cells

COS M6 cells in 10 cm dishes were transfected at ~50% confluency with 7 μg plasmid DNA/dish using the DEAE-dextran precipitation method [20]. After 48 h, cells were washed once in phosphate buffered

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saline, and lysed by sonication (10 s with probe tip) in 450 μl/dish of 0.5% (v/v) Triton X-100, 20 mM HEPES, pH 7.5, 2 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin. Following centrifugation (15 min, 20 000×*g*), the supernatant was adjusted to 200 mM NaCl and incubated with 25 μl of a 50% microcystin-Sepharose slurry (Upstate Biotechnology, Lake Placid, NY, USA) for 4 h at 4°C. Beads were washed 5× with 1.5 ml of 0.5% (v/v) Triton X-100, 200 mM NaCl, 50 mM Tris, pH 7.5, and proteins were eluted by boiling in SDS-PAGE sample buffer, followed by immunoblot analysis using alkaline phosphatase-conjugated secondary antibodies as described [21].

2.5. Immunofluorescence localization

COS M6 cells were grown on 22×22 mm glass coverslips in 35 mm dishes to ∼50% confluency and transfected with 2 μg/dish Bδ expression plasmid using TransIT-LT1 transfection reagent (Panvera Corp., Madison, WI, USA). After 48 h, cells were fixed and processed for immunofluorescence using affinity-purified Bδ antibody and Cy2-conjugated secondary antibody (Jackson Laboratories, West Grove, PA, USA) [22]. Phase contrast and fluorescence images were collected on a laser-scanning confocal microscope (Zeiss LSM 410) and processed using Adobe Photoshop software. Dishes transfected with empty vector served as negative control.

2.6. Subcellular fractionation

Adult rat brains (Harlan Bioproducts, Indianapolis, IN, USA) were fractionated into nuclear and cytosolic extracts as described [23]. To obtain cytosolic, low ionic strength (LISE), and membrane extracts, rat brains were thawed in 10 volumes Buffer A (100 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml leupeptin) and homogenized (5×10 s Polytron bursts). All manipulations were carried out at 4°C with pre-chilled solutions. The supernatant after an initial high-speed centrifugation (100 000×*g*, 1 h) was designated as cytosolic extract. The pellet was resuspended in 5 volumes (with respect to initial brain weight) Buffer A, centrifuged as above, and the supernatant was discarded. The resulting pellet was rehomogenized in 5 volumes Buffer B (1 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 1 μg/ml leupeptin), and dialyzed for 14–18 h against two changes of 20–30 volumes of Buffer B. The dialyzed homogenate was centrifuged (as above), and the resulting supernatant was termed LISE. The pellet was re-extracted by stirring in 5 volumes of 1% (v/v) Triton X-100 in Buffer B for 1–2 h, yielding a membrane extract as supernatant of a final high-speed centrifugation. Protein concentrations were determined using Bradford assay reagent (Biorad assay, Hercules, CA, USA) and bovine serum albumin as standard.

Protein phosphatases were purified from the extracts by microcystin-Sepharose precipitation (see Section 2.4) and immunoblotted for PP2A subunits. Blots were scanned and alkaline phosphatase reaction product was quantified by densitometry using NIH-image software (http://rsb.info.nih.gov/nih-image/).

3. Results and discussion

3.1. Sequence of a novel B subunit of PP2A

In an effort to identify novel members of the B subunit family of PP2A subunits, we synthesized primers corresponding to conserved regions of B subunits, and performed RT-PCR of adult rat brain RNA (see Section 2). In addition to

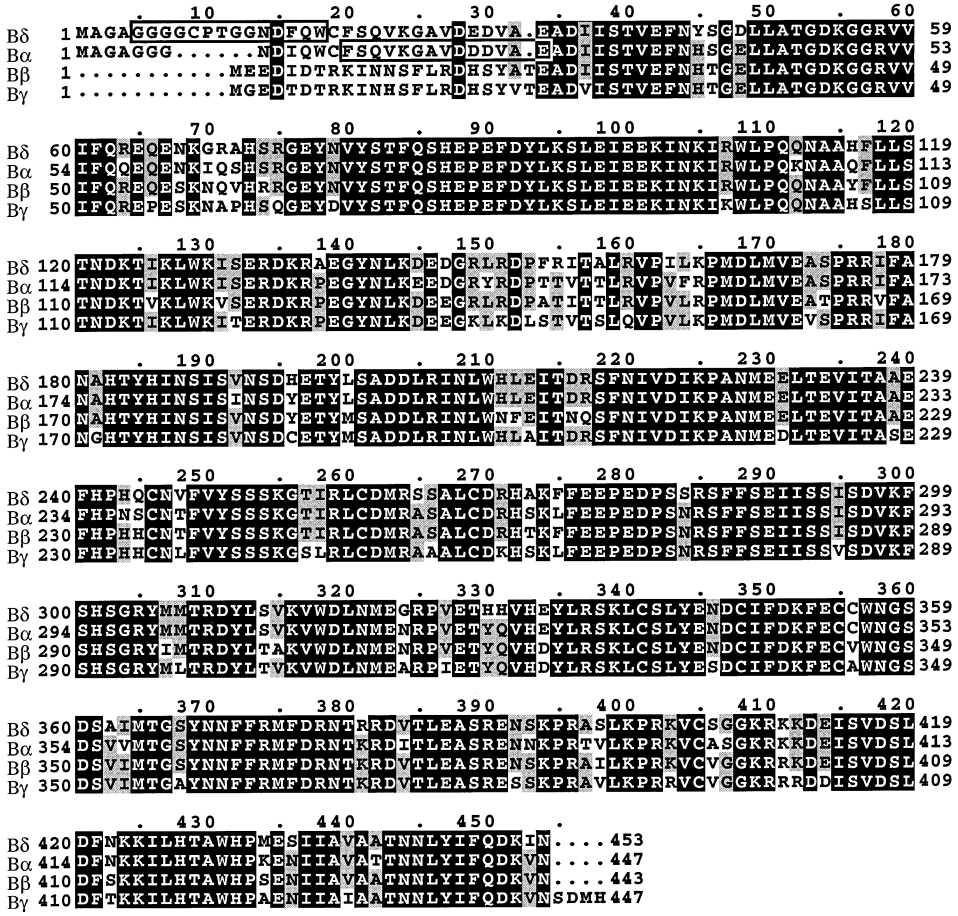

Fig. 1. Comparison of Bδ with other B subunits. The deduced amino acid sequence of Bδ was aligned with sequences of other B subunits from rat (Bα, [29]; Bβ, [30]; Bγ, [8]). Residues identical in all four isoforms are shaded in black; residues shared by three isoforms are shaded in grey. N-terminal sequences to which anti-peptide antibodies were generated are boxed.

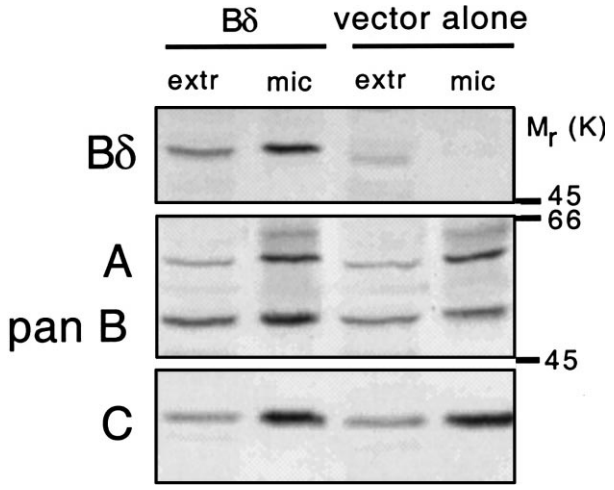

Fig. 2. Expression of Bδ cDNA as a functional phosphatase subunit. COS M6 cells were transfected with an expression vector containing the Bδ cDNA or vector alone. Cell extracts (extr, 20 μg/lane) and microcystin-Sepharose-bound proteins (mic) were immunoblotted with the antibodies indicated on the right. The position of molecular weight markers is shown on the left. This experiment is representative of three transfections.

identifying known B subunits, Bα, Bβ, and Bγ, this screen resulted in the isolation of multiple partial cDNA clones of a novel B subunit sequence. A 2034 bp cDNA was then isolated by a combination of 5′-RACE and cDNA library screening. Its single open reading frame predicts a novel protein of 453 amino acids and a molecular weight of 51 979, which was termed Bδ (GenBank accession number AF180350). The coding sequence of Bδ displays high sequence similarity to other B subunits (Fig. 1): at the protein level, Bδ is most identical to Bα (89%), followed by Bβ (83%), and Bγ (78%) (Fig. 1). Significantly, divergent residues are distributed throughout the sequence, suggesting strongly that Bδ is encoded by a separate gene.

Because of its divergence the extreme N-terminus of B subunits is a common target for the production of isoform-specific antibodies. However, residues in this region that have been used by us and others to raise Bα-specific antibodies (e.g. [18,24–26]) are highly conserved in Bδ. For instance, we previously generated antibodies to Bα 14–27 (Fig. 1) [18], which are identical in Bδ except for one conservative substitution (E for D23). It is therefore highly probable that available 'Bα' antibodies display mixed specificities for Bα and Bδ. Furthermore, since Bα and Bδ comigrate by one-dimensional SDS-PAGE (below), previous reports attributing a specific function to Bα should be reevaluated [19,25].

3.2. *Bδ is a phosphatase-associated protein*

While the absence of long runs of unique amino acids in Bα suggests it may be very difficult to generate Bα-specific antibodies, we succeeded in raising a Bδ antibody to a sequence including a 6 amino acid insert unique to Bδ (Fig. 1), which was used to characterize its expression.

To verify that the isolated cDNA indeed encodes a phosphatase subunit, COS M6 cells were transiently transfected with a Bδ expression plasmid or with a plasmid lacking insert, and then analyzed for PP2A subunit expression by immunoblotting (Fig. 2). COS M6 cells endogenously express Bα, but no detectable Bβ or Bγ [19]. A Bδ-immunoreactive protein of 53K molecular weight, close to the predicted molecular weight of 52K, was detected only in cells transfected with Bδ plasmid. This result demonstrates that the antibody is specific for Bδ and that COS cells express little or no endogenous Bδ. As detected by a pan B antibody [19], transfection of Bδ cDNA increased total B subunit amount compared to empty vector control (1.5 ± 0.1-fold, $N = 2$ transfections). A similar increase

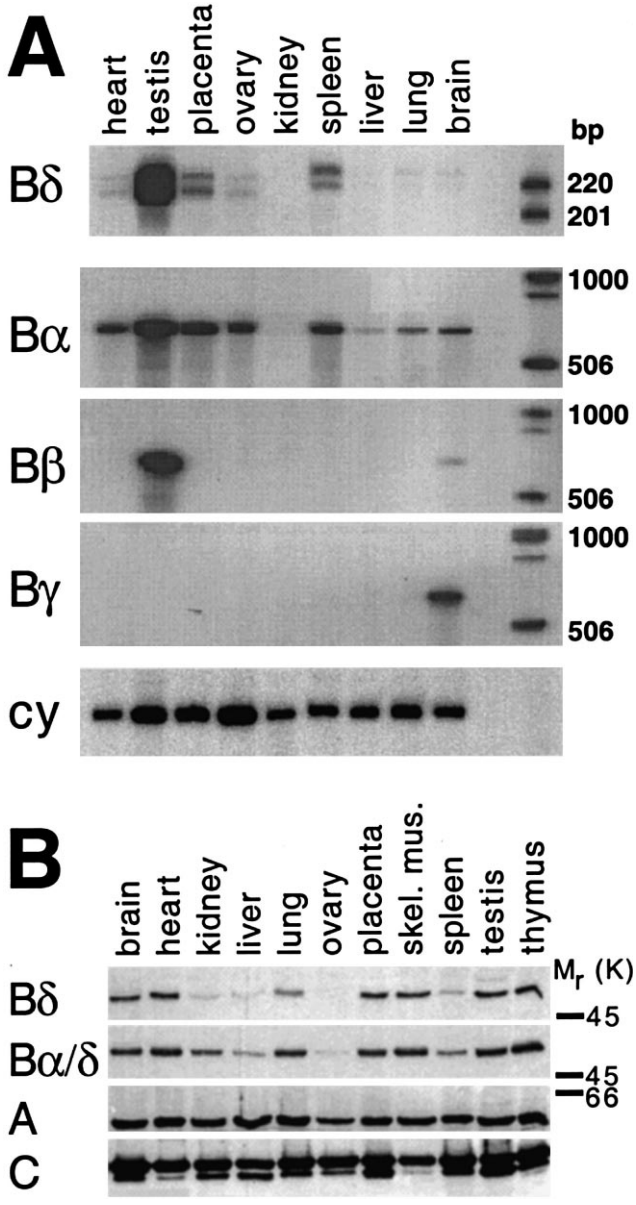

Fig. 3. Expression profile of PP2A subunits in rat tissues. A: Total RNA from the indicated rat tissues was analyzed by ribonuclease protection for expression of the B subunits indicated on the left; a cyclophilin (cy) probe served as an internal control for equal RNA loading. The position of size markers is indicated on the right. The protected Bδ RNA fragment migrates as a doublet, likely because of mismatches between Bδ mRNA and the degenerate primers used for amplifying the probe cDNA. B: Homogenates of the indicated rat tissues were solubilized in buffer containing 1% (v/v) Triton X-100 and clarified by centrifugation. Phosphatase holoenzymes were purified with microcystin-Sepharose from equal amounts of protein (600 μg/lane) and probed with antibodies indicated on the left. The position of molecular weight markers is shown on the right. Data in A and B are representatives of three experiments each.

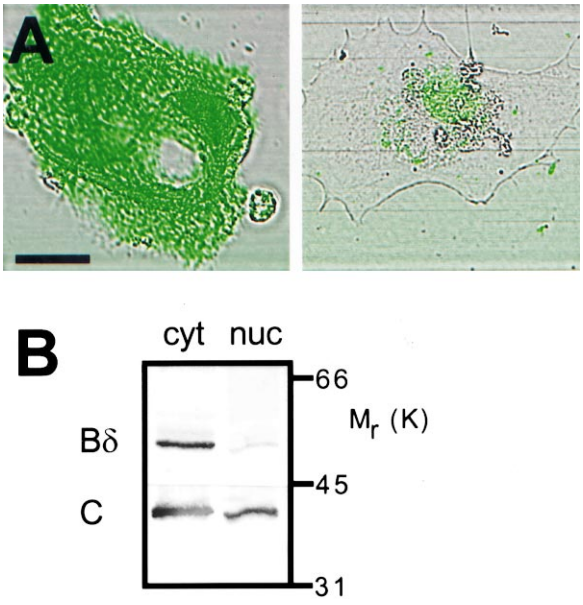

Fig. 4. Localization of Bδ to the cytosol. A: COS M6 cells were transfected with Bδ cDNA and analyzed by immunofluorescence with primary antibodies to Bδ and Cy2-conjugated secondary antibodies. Fluorescence images (green) were digitally merged with the corresponding phase contrast images to reveal outlines of cells and unstained structures. The cell on the left shows robust cytosolic Bδ immunofluorescence, whereas the cell on the right (from the same dish) was apparently not transfected and shows little staining. Scale bar: 25 μm. B: Rat brain proteins were fractionated into cytosol (cyt) and nuclear extract (nuc), purified with microcystin-Sepharose (from 800 μg protein/lane), and immunoblotted for Bδ and C subunit. Positions of molecular weight markers are shown on the right.

following Bδ transfection was seen with an antibody generated to Bα residues 14–27, indicating substantial crossreactivity of this antibody with Bδ (not shown). In contrast, levels of the constitutive A and C subunits remained unchanged.

The catalytic subunits of PP1 and PP2A bind tightly to the cyanobacterial toxin microcystin, and microcystin-Sepharose has been used to affinity-purify PP1 and PP2A holoenzymes [19,27,28]. Microcystin-Sepharose affinity purification of phosphatase subunits from extracts of transfected COS cells enriched Bδ to a similar extent as A and C subunits, indicating that Bδ is a bona fide PP2A subunit stoichiometrically complexed with A and C subunits in cells. Moreover, a peptide sequence matching Bδ uniquely was previously obtained by sequencing PP2A purified from skeletal muscle [29].

3.3. Tissue expression of Bδ mRNA and protein

One proposed role for B subunits is the regulation of PP2A activity and substrate selectivity in a cell- and tissue-specific fashion. Hence, the pattern of Bδ RNA and protein expression was examined in different rat tissues and compared to other PP2A subunits. For RNA detection, ribonuclease protection analysis was employed because of its high sensitivity and ability to faithfully discriminate between related transcripts. Bδ and Bα mRNA were detected in many tissues with a similar profile, with highest levels in testis (Fig. 3A). This profile contrasts with the much more restricted expression of Bβ RNA in testis and brain, and Bγ RNA in brain only, confirming previous Bβ and Bγ Northern [7,8,13,30] and immunoblot data [19].

A widespread, but not ubiquitous distribution of Bδ is also apparent at the protein level, with similarly high levels in brain, heart, placenta, skeletal muscle, testis and thymus (Fig. 3B). A comparison of relative levels of Bδ transcript and protein suggests that the Bδ mRNA in testis is not efficiently translated. A direct comparison of Bδ and Bα protein profiles is confounded by the lack of specific antibodies to Bα; antibodies previously raised to Bα detect Bδ as well (see Section 3.1). However, subtle differences in the staining pattern of the Bδ and Bα/δ antibodies suggest differences in the tissue distribution of the two B subunits: e.g. kidney, liver, and lung appear relatively deplete of Bδ compared to Bα.

3.4. Bδ targets PP2A to the cytosol

PP2A holoenzymes are distributed among many cellular compartments including cytosol, nucleus, membranes, microtubules and neurofilaments [18,19,21,25,31–33]. However, individual regulatory subunits show a more compartmentalized distribution [19,25,34] indicating a principal role of B subunits in targeting the PP2A heterotrimer to specific locations, thereby facilitating dephosphorylation of specific substrates. To localize Bδ-containing PP2A holoenzymes in the cell, COS M6 cells transfected with Bδ cDNA were analyzed by immunofluorescence (Fig. 4A). Bδ immunoreactivity was detected

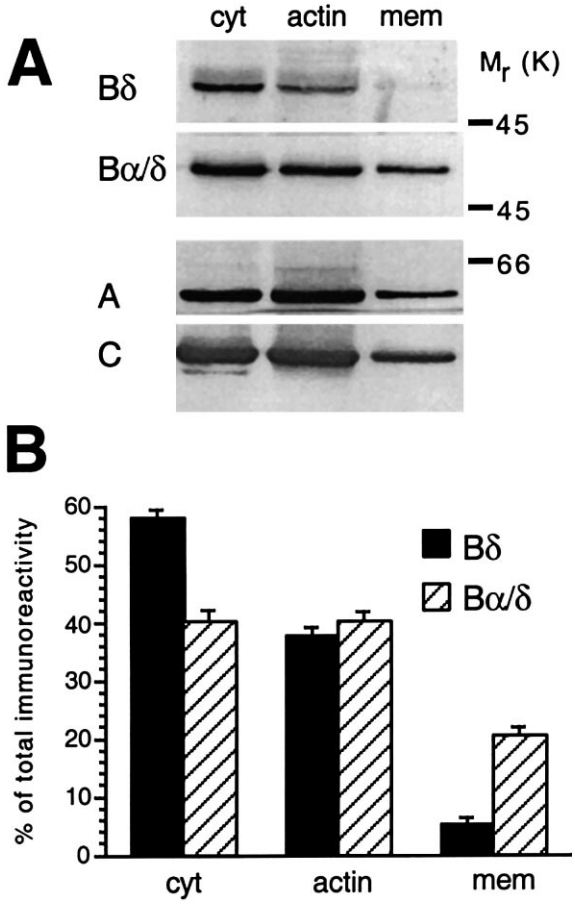

Fig. 5. Differential fractionation of Bδ and Bα. Brain proteins were fractionated into cytosol (cyt), an actin-cytoskeletal (actin), and a membrane (mem) extract, precipitated with microcystin-Sepharose (from 600 μg protein/lane), and immunoblotted for the indicated phosphatase subunits. A: Representative immunoblots; positions of molecular weight markers are shown on the right. B: Quantitation of Bδ and Bα/δ distribution in the three fractions as percent of total; plotted are means ± S.E.M. from three sets of extracts.

diffusely in the cytosol, excluding the nucleus. Little or no signal was found in non-transfected cells in the same dish (Fig. 4A, right) or in mock-transfected cells (not shown). To reveal the distribution of PP2A holoenzymes containing endogenous Bδ, rat brain proteins were fractionated into cytosolic and nuclear extracts and phosphatase holoenzymes were purified by microcystin affinity precipitation (Fig. 4B). While similar amounts of the catalytic subunit were detected in cytosolic and nuclear extracts, Bδ was highly enriched in the cytosol.

To further define the subcellular distribution of Bδ in comparison to its closest family member, Bα, rat brain proteins were fractionated into cytosol, a low ionic strength extract enriched in actin-cytoskeletal proteins [22,35], and a membrane protein fraction (Fig. 5). The analysis was restricted to protein fractions soluble under non-denaturing conditions (excluding, e.g. intermediate filaments and postsynaptic densities), in order to permit isolation of PP2A holoenzymes with microcystin-Sepharose, a concentration step required for reliable detection of Bδ (see Section 2). Most Bδ was present in the cytosolic fraction, less in the actin fraction, and almost undetectable amounts in the membrane fraction. This distribution differed significantly from the profile of combined Bα and Bδ protein detected with the dual specificity antibody, with equal amounts of Bα/δ in cytosol and actin fraction, and the remaining 20% in the membrane extract. While these data cannot be used to quantitate the exact amount Bα in each fraction, they indicate that a significant proportion of this subunit is associated with actin and membranes. Thus, even though Bα and Bδ display about 90% sequence identity, these two subunits appear to target the PP2A heterotrimer to different parts of the cell, presumably by associating with as yet unidentified binding proteins.

In conclusion, we have cloned Bδ, a novel member of the rapidly expanding family of PP2A regulatory subunits. Bδ is most closely related to Bα, with which it shares sequences previously used to generate supposedly subunit-specific antibodies. Bδ also shares with Bα a broad tissue distribution, but differs in its predominantly cytosolic distribution from Bα.

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