Heterotrimeric protein phosphatase 2A (PP2A) is a major Ser/Thr phosphatase composed of catalytic, structural, and regulatory subunits. Here, we characterize B\(\beta\), a novel splice variant of the neuronal B\(\beta\) regulatory subunit with a unique N-terminal tail. B\(\beta\) is expressed predominantly in forebrain areas, and PP2A holoenzymes containing B\(\beta\) are about 10-fold less abundant than those containing the B\(\beta1\) (previously B\(\beta\)) isoform. B\(\beta2\) mRNA is dramatically induced postnatally and in response to neuronal differentiation of a hippocampal progenitor cell line. The divergent N terminus of B\(\beta2\) does not affect phosphatase activity but encodes a subcellular targeting signal. B\(\beta2\), but not B\(\beta1\) or an N-terminal truncation mutant, colocalizes with mitochondria in neuronal PC12 cells. Moreover, the B\(\beta\) N-terminal tail is sufficient to target green fluorescent protein to this organelle. Inducible or transient expression of B\(\beta2\), but neither B\(\beta1\), B\(\gamma\), nor a B\(\beta\) mutant defective in holoenzyme formation, accelerates apoptosis in response to growth factor deprivation. Thus, alternative splicing of a mitochondrial localization signal generates a PP2A holoenzyme involved in neuronal survival signaling.

Reversible phosphorylation is a key post-translational regulatory mechanism in all eukaryotic cells. The phosphorylation state of any given protein is determined by the balance of protein kinase and phosphatase activities acting on it. Although it has long been appreciated that kinases assemble into complex signaling networks, our understanding of protein phosphatase regulation is comparatively limited. Protein phosphatase 2A (PP2A)\(^1\) is one of four major classes of Ser/Thr phosphatases (for a recent review, see Ref. 1). PP2A accounts for up to 1% of total protein in certain cell types, and together with PP1 it contributes greater than 90% of cellular Ser/Thr phosphatase activity (2–4). PP2A enzymatic activity is conferred by a ~36-kDa catalytic, or C subunit, which is highly conserved in evolution. Free C subunit is not known to exist in cells; rather it forms complexes with a variety of other proteins. The PP2A core dimer is composed of the C subunit and the scaffolding A (or PR65) subunit, and several other complexes containing one, but not the other subunit have also been described (5–7).

The predominant form of PP2A, however, is the trimeric holoenzyme consisting of the core dimer complexed to a third variable regulatory subunit. In mammals, regulatory subunits are encoded by four gene families denoted B (or PR55), B\(^2\) (PR61, B56), B\(^\gamma\) (PR48, PR59, PR72/130), and B\(^\delta\) (striatin, S2GNA). Proposed functions of these subunits include regulation of catalytic activity, substrate specificity, and subcellular localization of PP2A. The PP2A B subunit family has four members (B\(\alpha\)–\(\delta\)). The five B subunit genes (B\(\alpha\)–\(\epsilon\)) encode phosphoproteins with diverse functions including regulation of Wnt/β-catenin signaling (8, 9). The B\(\alpha\) family consists of four polypeptides that arise from three genes (PR72/130, PR48, PR59). B\(^2\) subunits are nuclear proteins that bind calcium and have been implicated in the regulation of the G1/S cell cycle transition (10–12). Recent RNA interference Studies in Drosophila cells have demonstrated that B family subunits regulate mitogen-activated protein kinase signaling, whereas B\(\gamma\) subunits protect cells from apoptosis (13, 14).

Even though they were the first PP2A regulatory subunits to be identified, few functions of the mammalian B family have been uncovered to date. Structurally, B family subunits resemble β subunits of heteromeric G proteins in that they contain seven WD repeat motifs predicted to fold into a β-propeller (15). B\(\alpha\), the most abundant B family member, is expressed in a variety of cell types and mediates dephosphorylation of the cytoskeletal proteins tau and vimentin by PP2A (16–18). The recently identified B\(\gamma\) subunit is most similar to B\(\alpha\) and is also expressed in multiple tissues (19). B\(\beta\) and By genes, on the other hand, give rise to neuron-specific members of the B family of PP2A subunits with distinct temporal and spatial expression patterns in brain (20). Forced expression of B\(\gamma\), but not other PP2A regulatory subunits, promotes neuronal differentiation of PC12 cells, an effect that appears to be mediated by activation of the mitogen-activated protein kinase cascade at the level or upstream of the Ser/Thr kinase B-Raf (21).

An important role of B\(\beta\) in neuronal survival was suggested by the discovery that the neurodegenerative disorder spinocerebellar ataxia type 12 is caused by a trinucleotide repeat expansion in the promoter region of the human B\(\beta\) gene (PPR2R2B) (22). Thus, dysregulated B\(\beta\) gene expression may be detrimental to neurons, ultimately leading to the massive cerebral and cerebellar atrophy seen in spinocerebellar ataxia type 12 patients. In this report, we characterize a novel splice product of the B\(\beta\) gene which is induced upon neuronal differentiation. The unique N-terminal extension of B\(\beta2\) is shown to

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\(^1\) The abbreviations used are: PP2A, protein phosphatase 2A; EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; GFP, green fluorescent protein; RT, reverse transcription; UTR, untranslated region.

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target the protein to mitochondria, where B2B accelerates neuronal cell death after survival factor deprivation.

EXPERIMENTAL PROCEDURES

Isolation of the B2B cDNA—Duplicate filters containing 1 × 10⁷ plaque-forming units from a rat brain cDNA library in the λZap II vector (Stratagene) were screened with a random primed, [α-³²P]dCTP-labeled probe corresponding to the full-length mouse B1 cDNA (a gift from Dr. Nat Heintz, Rockefeller University). After four rounds of screening, a partial cDNA containing the 5'-UTR and the N-terminal third of the B2B coding sequence was isolated. The full-length coding sequence for B2B was obtained by reverse transcription-PCR (RT-PCR) from total rat brain RNA with primers complementary to the unique 5'-coding sequence and common 3'-UTR (5'-coding sequence/forward primer: 5'-AAA TGC TTC TTC CGT TAC CT-3'; 3'-UTR/reverse primer: 5'-GGT TTG ACT AGT ATT CAG TAT GTG-3'). The B2B cDNA sequence was submitted to GenBank and is available under accession number AY251277.

Generation of FLAG- and Green Fluorescent Protein (GFP)-tagged Bβ Constructs and Site-directed Mutagenesis—Primers complementary to the N terminus of Bβ1 and Bβ2 and to the beginning of the common region (TEAD) fitted with a HindIII cloning site in conjunction with two nested reverse primers including sequence complementary to the Bβ C-terminus were used. SLAM epitope tag, and SLAM cloning site were used to PCR amplify Bβ1, Bβ2, and Bβ3N, respectively. PCR fragments were ligated into pcDNA5/TO or pEGFP-N1 to generate fusion proteins with C-terminal FLAG and FLAG-GFP sequences, respectively. Bβ1–123 GFP and Bβ2–130 GFP were constructed by excising C-terminal sequences from the full-length Bβ1/2-pEGFP-N1 plasmids by EcoRI/Xmal digestion, filling in the overhangs with Klenow polymerase, and religating the plasmids. The Bβ2 RR168EE mutant was constructed by full plasmid synthesis using Phus Ultra polymerase according to instructions for the QuikChange mutagenesis kit (Stratagene). All constructs were fully sequenced at the University of Iowa DNA Facility.

Antibodies—A peptide derived from the N terminus of Bβ2 (CF8-1289) was used to couple to keyhole limpet hemocyanin via the sulphydryl group of the N-terminal cysteine, and polyclonal antibodies were generated in rabbits and affinity purified by standard techniques (23). Bβ1 and pan-B subunit antibodies have been described previously (20). Monoclonal antibodies to the PP2A A subunit were a kind gift from Gernot Walter (University of California San Diego), and PP2A C subunit antibodies were purchased from Transduction Laboratories. The adenine nucleotide translocase antibody was provided by Harmut Wohlrab (Boston Biomedical Research Institute).

Ribonuclease Protection Analysis—The Bβ2 cDNA library clone was subcloned into pBluescript KS+ and in vitro transcribed using T7 polymerase. Total RNA was isolated from selected rat organs and brain regions using Trizol reagent according to the manufacturer’s instruction (Molecular Research Center). Ribonuclease protection was carried out as described previously (20, 24).

Competitive RT-PCR—Total RNA (0.5–1.0 μg) was reverse transcribed and PCR amplified in the same 25-μl reaction with reagents from the Access RT-PCR kit (Promega, Madison, WI) and the following primers (0.5 μM each): common reverse, 5′-GAC ATC AAG CCA GCC AAC ATG GAG G-3′; Bβ1 forward, 5′-TGC CCC CTC CTC TGT TGA GAC-3′; Bβ2 forward, 5′-ACC ATC CTC TCC AGC TGC C-3′. Aliquots of PCR were separated on 1% agarose gels and ethidium bromide-stained bands were quantified by image analysis using NIH Image software. The ratio of the 749-bp Bβ1 and 619-bp Bβ2 PCR products was found to be independent of the number of PCR cycles; 5 cycles were routinely used.

Cell Culture—COS-M6 and PC6-3 cells were cultured and transfected as described previously (15, 21). The adult hippocampal progenitor cell line HC2S2 was generously provided by Fred Gage (Salk Institute) and cultured for 72 h in regular growth medium (10% horse serum, 5% fetal bovine serum in RPMI 1640) in the presence of vehicle (0.1% ethanol) or 1 μM/3 mg/ml doxycycline. After two washes, serum-free RPMI 1640 + doxycycline was added, and cell density was assessed by MTS tetrazolium reduction to formazan according to the manufacturer’s instructions (CellTiter 96® AQueous nonradioactive cell proliferation assay, Promega). Formazan production was quantified after 48 h incubation at 490 nm using a 96-well plate reader. The MTS assay was repeated after 24 h in serum-free medium, and cell survival was expressed as the ratio of the two measurements. Previous apoptosis studies with PC6-3 cells have documented excellent correlation between cell counts and metabolic activity as assessed by tetrazolium salt reduction (27).

For nuclear morphology assays, native PC6-3 cells or tetracycline-inducible cells were seeded at 200,000 cells/well in 20-mm² chamber cover glasses. Native PC6-3 cells were transiently transfected with 1 μg/chamber GFP fusion protein plasmids using LipofectAMINE 2000 and cultured for 48 h, whereas inducible cells were treated with vehicle (0.1% ethanol) or 1 μg/ml doxycycline for 72 h pre-incubation and 48 h under serum-free conditions, cultures were fixed in 3.7% paraformaldehyde in phosphate-buffered saline, incubated with the blue fluorescent nuclear stain Hoechst 33342 at 1 μg/ml for 5 min and mounted on slides. Random microscopic fields (6–12 fields/culture, 50–200 cells/field) were counted.
24 amino acids. The full-length cDNA for B2/H9252 upstream of the initiation codon (30), the B2 is shown in Fig. 1. The gene structure of the human and B2/H9252 by exons separated by irregular, or fragmented nuclei were scored as apoptotic. Therefore, we performed ribonuclease protection assays with probes corresponding to the divergent domains to map the relative abundance of B2 and displayed no cross-reactivity with Bβ1 or other PP2A regulatory subunits (Fig. 2, and not shown). Although Bβ1 could be detected in total brain lysates (Ref. 20 and Fig. 2), antibody detection of a protein with the size predicted for Bβ2 (52,000) necessitated enrichment of PP2A holoenzymes by microcystin-Sepharose affinity purification (20). COS cell lysates expressing FLAG epitope-tagged Bβ splice variants were used as standards and immunoblotted with Bβ isomorph-specific and FLAG-directed antibodies to compare detection strengths of Bβ1 and Bβ2 antibodies. Thus normalizing for antibody affinities and titers, the relative abundance of Bβ1- and Bβ2-containing PP2A holoenzymes in rat brain was estimated to be ~10:1.

The low abundance of Bβ2 precluded an analysis of its spatial and temporal expression pattern at the protein level. Therefore, we performed ribonucleic acid protection assays with probes corresponding to the divergent domains to map the expression of Bβ isoforms in rat brain regions. Bβ1 and Bβ2 transcripts were detected at comparable levels in all forebrain

![Diagram](image-url)
structures, except in olfactory bulb, where relatively more B1 was expressed (Fig. 3 and Ref. 20). The cerebellum contained low levels of both B2 splice forms.

We reported previously that B family regulatory subunits exhibit distinct developmental expression profiles in brain (20). The neuronal B1 isoform is induced during postnatal brain development, whereas Bα and Bβ show constant expression and a slight postnatal decline in expression, respectively. Ribonuclease protection assays with a B2-specific probe revealed that this isoform has an expression pattern similar to Bγ, with near undetectable expression at birth rising to adult levels by postnatal day 14 (Fig. 4A). Thus, alternative promoter use and splicing of the Bβ gene appear to be regulated developmentally.

We developed a competitive RT-PCR protocol to assay changes in relative abundance of Bβ1 and Bβ2 transcripts rapidly. In this assay, reverse transcription of mRNA is carried out with probes that correspond to the unique 5'-UTR and N-terminal coding regions of Bβ1 and Bβ2. A, total RNA from the indicated organs of adult rats (Br, brain; Lu, lung; Li, liver; Sp, spleen; Ki, kidney; Ov, ovary; Pl, placenta; Te, testis; He, heart) was analyzed for Bβ2 and cytochrome c, internal control transcript levels. B, total RNA from the indicated brain regions of adult rats (St, striatum; OB, olfactory bulb; Mi, midbrain; Hi, hindbrain; Di, diencephalon; Co, cortex; BS, brain stem; Ce, cerebellum) was subjected to ribonuclease protection analysis. The graph shows Bβ1 and Bβ2 densities normalized to the cyclophilin internal control and to the average of each series (means ± S.E. of three sets of RNA preparations). The Bβ1 expression data are replotted from Ref. 20 to facilitate comparison. A representative autoradiogram of Bβ expression is shown at the bottom.

Our data indicate that Bβ2 mRNA is found specifically in mature brain, but do not rule out a non-neuronal (e.g. glial) origin of expression. To address this issue, multiple cell lines of neuronal (PC6-3, PC12, B104, SHSY5Y, Neuro2A), glial (C6, Ng108), and other (COS, HEK293, NIH3T3, MCF7) origin were analyzed for Bβ isoform expression by competitive RT-PCR. Although all neuronal cell lines tested expressed Bβ1, none had detectable levels of Bβ2 (data not shown). To examine alternative splicing of the Bβ locus in a cell line that more closely resembles primary forebrain neurons, we turned to HC2S2 cells, a neuronal progenitor cell line derived from rat hippocampus (25). HC2S2 cells are conditionally immortalized by a tetracycline-repressible v-myc oncogene and differentiate into phenotypic neurons upon addition of tetracycline or doxycycline to the medium (Fig. 4D). Competitive RT-PCR with Bβ1 and Bβ2 primers was performed on HC2S2 cultures treated for up to 4 days with doxycycline. Bβ2 mRNA was already detectable in dividing HC2S2 cells, and levels increased further relative to Bβ1 as cells differentiated into neurons (Fig. 4E). These data strongly indicate a neuronal locus of Bβ2 expression. The time course of Bβ isoform expression in differentiating HC2S2 cells closely parallels that seen in postnatal maturation of the brain (compare Fig. 4, C and E). With the caveats inherent to a comparison between neuronal differentiation in vitro and brain development in the intact organism, these data suggest that the HC2S2 cell line may be an appropriate model system for Bβ gene regulation studies.

The Bβ2 N Terminus Does Not Affect Holoenzyme Formation or Catalytic Activity—Previous structure-function studies indicated that the variable N terminus of Bγ is inherent to a comparison between neuronal differentiation and a non-neuronal (e.g. catalytic) role. To investigate whether the differentially spliced N termini of Bβ1 and Bβ2 play a role in formation or catalytic activity of the PP2A holoenzyme, the two isoforms were FLAG epitope tagged at the C terminus and transiently expressed in COS-M6.
cells. A deletion mutant lacking the divergent N terminus, BβΔN, was also constructed and analyzed in parallel. The ectopically expressed proteins were immuno-isolated with anti-FLAG resin and analyzed for association with endogenous A and C subunits by immunoblotting. Bβ1, Bβ2, and BβΔN could be expressed to similar levels and associated with equivalent amounts of A and C subunits (Fig. 5A). Aliquots of the immunoprecipitates were then assayed for dephosphorylation of two model substrates, myelin basic protein and casein phosphorylated in vitro by protein kinase A (Fig. 5B). Myelin basic protein was a better substrate than the more acidic casein in these assays. Importantly, the three PP2A heterotrimers had equivalent activities toward these substrates. Therefore, we conclude that the divergent N termini of Bβ isoforms are not involved in formation of the PP2A heterotrimer and do not influence substrate recognition, at least in these in vitro assays.

The Bβ2 N Terminus Encodes a Mitochondrial Localization Signal—Bα, Bβ1, and Bβ2 are regulated developmentally in the hippocampal progenitor cell line HC282 in the dividing state (left) and 4 days after addition of doxycycline (Dox) to induce neuronal differentiation (right). E, competitive RT-PCR analysis of relative expression levels of Bβ1 and Bβ2 mRNA in HC282 cells treated for the indicated days with doxycycline.

**Fig. 5. In vitro characterization of PP2A holoenzymes containing Bβ isoforms. A, FLAG epitope-tagged Bβ1, Bβ2, or a truncation mutant lacking the divergent N terminus (BβΔN) was transiently expressed in COS-M6 cells; transfections with empty vector served as controls. FLAG immunoprecipitates (IP) were probed for transfected Bβ subunits and endogenous A and C subunits. B, PP2A holoenzymes containing the indicated FLAG-tagged Bβ subunits were immuno-isolated as in A and assayed for activity toward exogenous, 32P-labeled substrates (protein kinase A-phosphorylated myelin basic protein, MBP; protein kinase A-phosphorylated casein). Shown are the means ± S.D. of duplicate determinations.
signal that overrides a “default” address in the common region of Bβ for punctate localization. The latter interpretation can be discounted because the subcellular distribution the N-terminal deletion mutant BβΔN was indistinguishable from Bβ1-GFP (Fig. 6A).

The puncta labeled by Bβ2-GFP were identified as mitochondria in double labeling experiments with the red rosamine derivative dye MitoTracker, which accumulates in actively respiring mitochondria (Fig. 6B). Bβ1-GFP, in contrast, appeared deplete in areas with high densities of mitochondria.

To investigate whether the Bβ2 N terminus is sufficient for targeting to mitochondria, the first 35 amino acids of Bβ2,
including the unique 24 residues and 11 residues shared with Bβ1, were fused to the N terminus of GFP (Bβ21–35-GFP). The corresponding N-terminal fusion of Bβ1 (Bβ11–32-GFP) served as a control. The N terminus of Bβ2, but not Bβ1, was capable of targeting GFP to mitochondria in PC6-3 cells (Fig. 6C). In contrast to the full-length protein, Bβ21–35-GFP showed a strikingly discrete mitochondrial localization in virtually every transfected cell, with little if any diffuse fluorescence. It is conceivable that the common C-terminal region of the Bβ splice variants associates with cytoplasmic proteins/structures, which gives rise to the mixed diffuse/mitochondrial localization of full-length Bβ2-GFP.

Mitochondrial localization of the Bβ2 N terminus was also demonstrated by subcellular fractionation. Transient expression of Bβ21–35-GFP gave rise to two GFP immunoreactive bands with mobilities of 31,000 and 33,000; the predicted molecular weight is 31,727 (Fig. 7). This heterogeneity may be a consequence of proteolysis, internal translation initiation, or post-translational modification. The lower mobility, presumably full-length or post-translationally processed Bβ2 N-terminal fusion protein cofractionated with mitochondria, whereas the smaller protein and the Bβ1 N terminus (Bβ11–32-GFP) were mostly soluble.

Bβ2 Promotes Apoptosis—In addition to performing critical functions in biosynthesis and energy metabolism, mitochondria are central to apoptotic signal transduction (31). To explore a possible function of mitochondria-targeted Bβ2 in neuronal apoptosis, we generated a panel of stable, clonal PC6-3 cell lines that express Bβ1, Bβ2, or Bγ by under control of a tetracycline-inducible cytomegalovirus promoter (21). The PC6-3 subline of PC12 cells was established by Pittman and coworkers (27) as a neuronal apoptosis model that more closely resembles sympathetic neurons than the parental PC12 cell line. Undifferentiated PC6-3 cells express primarily Bα, whereas nerve growth factor-differentiated cells additionally express Bβ1 and Bγ (21). Endogenous Bβ2 expression is undetectable by RT-PCR under either condition (data not shown).

Growth of the stable PC6-3 cell lines in doxycycline-containing medium led to the induction of comparable levels of Bβ1, Bβ2, and Bγ, as detected with an antibody to the FLAG epitope tag (Fig. 8A). Approximately 2-fold overexpression was achieved over the endogenous Bα subunit, as visualized with a pan-B subunit antibody. Levels of A and C subunits, as well as levels of members of the B′ regulatory subunit family, were unaltered following B subunit induction (Fig. 8A and data not shown). Growth rates were unaffected by doxycycline treatment in two independently isolated Bβ2-expressing cell lines (data not shown). We also assayed cell viability in serum-containing medium and found that Bβ2 induction is not toxic to cells (Fig. 8B).

Complete removal of serum kills 20–50% of PC6-3 cells within 24 h as assayed by tetrazolium salt reduction (Fig. 8C). In two different clonal cell lines, inducible Bβ2 expression decreased survival by 30–40% assayed 24 h after serum withdrawal (Fig. 8C). Accelerated cell death was specific for this mitochondria-localized Bβ splice variant because Bβ1 or Bγ induction had little to no effect on cell survival. Nuclear condensation and fragmentation are hallmarks of late stage apoptosis. We examined nuclear morphology after staining with a DNA dye to demonstrate that Bβ2 decreases survival by promoting apoptosis. Inducible expression of Bβ2, but not Bβ1, almost doubled the number of cells with apoptotic nuclei after 24 h in serum-free medium (Fig. 8D).

Bβ2 Requires Incorporation into the PP2A Heterotrimer to Promote Apoptosis—It is conceivable that binding of the Bβ2 N terminus to mitochondria has a nonspecific toxic effect on cells. To address this issue, we carried out apoptosis experiments in which various B family regulatory subunit constructs tagged at the C terminus with GFP were transiently transfected into PC6-3 cells. 24 h after serum removal, GFP-positive cells with apoptotic nuclei were counted. In agreement with the data from inducible cell lines, we found that transient expression of Bβ2, but not Bβ1 or Bγ, increased the number of apoptotic cells compared with transfection with GFP alone (30% versus 5%, Fig. 9A).

Arg-165 and Arg-166 of Bγ form critical salt bridges with Glu-100 and Glu-101 of the Aα subunit (15). We replaced the corresponding pair of arginines in Bβ2 with glutamates to generate a mutant (RR166EE) that cannot associate with the AC dimer (Fig. 9B). Bβ2 RR166EE was able to bind efficiently to an Aα subunit carrying the opposite charge reversal mutation (EE100RR), demonstrating that the mutant Bβ2 protein folds normally (data not shown). As expected, we also failed to detect binding of PP2A A and C subunits to the mitochondria-targeting N terminus of Bβ2 (Bβ21–35, Fig. 9B). Neither the monomeric Bβ2 point mutant nor the Bβ2 N terminus fused to GFP was able to promote apoptosis after growth factor deprivation in transient transfection assays (Fig. 9, C and D) even though fluorescence levels were equivalent to (Bβ2 RR166EE, Fig. 9C) or much greater (Bβ21–35) than full-length, wild-type Bβ2. These data strongly support a model in which Bβ2 modulates neuronal survival by targeting an active PP2A heterotrimer to dephosphorylate mitochondrial substrates.

DISCUSSION

Protein kinases recognize their substrates via primary sequence motifs surrounding phosphorylatable residues. In addition, the spatial constraint imposed by tethering protein kinases to organelles and other subcellular structures further enhances the fidelity of intracellular signaling via these enzymes (32). In contrast, consensus sequences appear to contribute little to substrate recognition by protein phosphatases (33), and mechanisms for specific subcellular targeting of these enzymes remain relatively unexplored. This report challenges the view that phosphatases lack specificity by documenting the first example of a mitochondria-localized protein phosphatase subunit.

We show that the gene for the Bβ regulatory subunit of PP2A gives rise to an alternative splice variant, termed Bβ2. Bβ2 mRNA is highly expressed in forebrain structures and is induced during postnatal brain development and during differentiation of an adult hippocampal progenitor cell line. The unique N-terminal extension of Bβ2 is shown to be necessary and sufficient for targeting to mitochondria. A functional consequence of mitochondrial localization appears to be modula-
of apoptosis, as demonstrated by transient and inducible overexpression of Bβ2 in a neuronal cell line.

**Complexity of Bβ Gene Expression**—The Bβ gene (PPP2R2B) is unique among genes encoding B family PP2A regulatory subunits in that it gives rise to multiple variants. In a recent report, Schmidt et al. (29) reported the cloning of cDNAs for two novel murine Bβ isoforms: Bβ.2 is the murine ortholog of Bβ2, the subject of the present report. Bβ.1, which has a distinct N-terminal tail, may be a murine-specific Bβ splice form because no orthologs are present in other EST data bases, and RT-PCR failed to detect the presence of this isoform in rat brain.2 The number of potential Bβ isoforms appears to be even greater because RT-PCR from human brain samples combined with EST and genome data base searches identified several other Bβ gene transcripts that have unique 5′-UTR and N-terminal sequences.3 Judging by the number of entries for Bβ2 in human and mouse EST data bases, Bβ2 appears to be the second most common Bβ isoform, although it is much less abundant than Bβ1 at the protein level (Fig. 2). It seems therefore likely that the remaining, uncharacterized Bβ isoforms are expressed at extremely low levels or in only small subsets of neurons.

Bβ has recently attracted the attention of the research community because of its involvement in the neurodegenerative disorder spinocerebellar ataxia type 12. A CAG trinucleotide repeat expansion immediately upstream of the transcription initiation site of Bβ1 was found to be responsible for this disorder (22), which is a relatively common type of spinocerebellar ataxia in India (34). It will be important to address the effect of this repeat expansion on mRNA and protein levels of not only Bβ1, but also other Bβ isoforms, especially in light of our finding that Bβ2 overexpression promotes neuronal apoptosis.

**Structural Implications**—Members of the B family of PP2A regulatory subunits are greater than 80% identical at the amino acid level, with greatest sequence divergence at the N terminus. These proteins are predicted to adopt a toroidal, β-propeller structure that makes multiple contacts with the AC dimer (15). Pairs of conserved arginines that bind directly to a adjacent glutamates in the A subunit, as well as other amino acids critical for holoenzyme association map to WD repeats 3 and 4 in the mid portion of the B subunit molecule. Based on these data, we arrived at the model topology shown in Fig. 1B. The divergent N terminus is located opposite the AC dimer interface, where it is free to engage in macromolecular interactions that determine the subcellular localization of the PP2A holoenzyme.
Consistent with this model and our previous mutagenesis data with Bγ (15), we find that the N-terminal tail can be deleted from Bβ without disrupting the holoenzyme (Fig. 5A). Furthermore, neither the presence nor the identity of the N-terminal tail has any effect on phosphatase activities toward two model substrates (Fig. 5B), arguing that the divergent residues are not involved in direct binding to substrates. Instead, the N terminus of Bβ was found to encode a subcellular targeting module that can direct GFP to mitochondria as shown by microscopy and biochemistry (Figs. 6 and 7). By analogy, we propose that the differential localization of other B family subunits (20) is also a function of their N-terminal sequences.

How the Bβ2 N terminus interacts with mitochondria is unknown at present. Most nuclear encoded proteins destined for the mitochondrial matrix contain N-terminal sequences that are cleaved by signal peptidases (35). If proteolysis of the Bβ2 N terminus occurs at all, it is restricted to the cytosol (Fig. 7). In addition, mitochondrial protein import involves unraveling of tertiary structure (36), which would be incompatible with holoenzyme association of Bβ2. Because of these considerations, we hypothesize that the Bβ2 N terminus binds to a protein or lipid constituent of the outer mitochondrial membrane. Bβ2 may be targeted to mitochondria in a manner similar to hexokinase I, whose N terminus binds to the outer mitochondrial membrane protein porin (37). Alternatively, the Bβ2 N terminus may interact with mitochondrial lipids such as cardiolipin (38), possibly subsequent to acylation of specific residues. For instance, mitochondria association of the small GTPase Rab32 is thought to depend on fatty acid modification of two cysteine residues near its C terminus (39). Inspection of the Bβ2 N terminus does not reveal any sequence similarities to other outer mitochondrial membrane targeting sequences (37, 40, 41), but it is noteworthy that two cysteine residues at positions 3 and 23 are conserved in mammalian and fish Bβ2 orthologs.

**PP2A in Apoptosis**—The pheochromocytoma PC12 cell line and its PC6-3 subline are established model systems for neuronal differentiation and apoptosis studies (27, 42). We have generated stable, clonal PC6-3 lines that express neuronal PP2A regulatory subunits under control of a tetracycline-inducible promoter to investigate their involvement in apoptosis signal transduction pathways. Inducible expression levels of neuronal regulatory subunits are similar to the endogenous Bo subunit and are not accompanied by any changes in A, C, or other regulatory subunit levels (Fig. 5A). The lack of any compensatory changes in combination with the known instability of monomeric B family regulatory subunits (15) suggests that the induced regulatory subunits complex to a pool of free PP2A dimer in the cell (43). Using this system, we show that inducible expression of the mitochondria-targeted Bβ2 subunit potentiates neuronal death after growth factor withdrawal without affecting cell viability in the presence of serum (Fig. 8). It is important to point out that Bβ1, Bβ2, and Bγ induction levels in PC6-3 cell lines are likely considerably higher than in native neurons, where Bγ is the most abundant B family regulatory subunit (20). Demonstrating convincingly that endogenous Bβ2 is proapoptotic will necessitate knocking down its expression in neurons by gene targeting or RNA interference techniques.

The decision between cell survival and apoptotic cell death depends on relative expressions levels and phosphorylation states of pro- and antiapoptotic members of the BCL-2 family of proteins (31). We hypothesize that targeting of PP2A to mitochondria via the divergent N terminus of Bβ2 tips the balance toward phosphorylation, facilitating the activation of pro-apoptotic proteins or the inactivation of antiapoptotic proteins when cells are challenged by removal of survival factors.

Several lines of evidence support the idea that the balance of kinase and phosphatase activities at the mitochondrial membrane is pivotal for survival signaling. In a set of experiments complementary to the present study, Affaitati et al. (44) showed...
that inductive overexpression of the mitochondria-targeted A kinase anchoring protein (AKAP) 121 promotes survival of PC12 cells. The prosurvival effect of AKAP121 was suggested to involve enhanced phosphorylation by protein kinase A and cytosolic sequestration of BAD, a proapoptotic BCL-2 family protein. Significantly, a PP2A-like activity was implicated in BAD dephosphorylation and apoptosis of lymphoid cells after interleukin-3 removal (45). In another set of studies with a leukemia-derived cell line, toxic concentrations of the lipid second messenger ceramide were shown to activate PP2A, promote its translocation to the mitochondrial membrane, and cause dephosphorylation of the prosurvival protein BCL-2 at Ser-70 (46, 47). A member of the B’ family of PP2A regulatory subunits highly expressed in non-neuronal tissues, B’α, was implicated in targeting PP2A to BCL-2 in the latter studies, suggesting that induction of apoptosis may involve multiple PP2A holoenzymes. Identifying the mitochondrial substrates of the Bβ2-containing PP2A holoenzyme is a goal of ongoing experiments and should provide further insights into the function of Bβ2 in neurons and possibly the etiology of spinocerebellar ataxia type 12.

The observation that Bβ2 is induced during postnatal brain development (Fig. 4) is intriguing in the context of the increased vulnerability of the aging brain to a variety of stressors and insults (48). Inhibition of Bβ2 expression or subcellular targeting may therefore provide an attractive avenue for the treatment of brain injuries and neurodegenerative disorders.

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