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Molecular Determinants for PP2A Substrate Specificity: Charged Residues Mediate Dephosphorylation of Tyrosine Hydroxylase by the PP2A/B′ Regulatory Subunit†

Amit Saraf, Elizabeth A. Oberg, and Stefan Strack*

Department of Pharmacology, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242

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ABSTRACT: Together with protein phosphatase 1, protein phosphatase 2A (PP2A) contributes the bulk of Ser/Thr phosphatase activity in most cell types. The predominant form of PP2A is a heterotrimer of catalytic (C), scaffolding (A), and diverse regulatory subunits (B, B′, and B″). We have previously shown that N-terminal phosphorylation sites in tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, are specifically dephosphorylated by PP2A holoenzymes containing the B′β regulatory subunit. Here, we identify a Glu residue conserved in B′ regulatory subunits that is critical for dephosphorylation and inactivation of tyrosine hydroxylase in vitro and in PC12 cells. According to the PP2A heterotrimer crystal structure, Glu153 (B′β numbering) abuts the catalytic site on the C subunit, and we demonstrate that Glu153 substitution inhibits multisite TH dephosphorylation without compromising PP2A/B′β holoenzyme assembly or in vitro dephosphorylation of model substrates. Apart from its role in modulating TH activity, Glu153 is also necessary for PP2A/B′β-mediated enhancement of nerve growth factor signaling. Furthermore, global phosphoproteome analysis suggests that Glu153 mediates dephosphorylation of most B′β substrates in PC12 cells. With regard to selectivity determinants in the substrate, we show that B′β Glu153 recognizes Arg37 and Arg38 in TH to direct dephosphorylation of both upstream (Ser31) and downstream (Ser40) sites. These results provide evidence of a subunit-spanning substrate docking site on the PP2A/B′ holoenzyme, in which negatively charged side chains in the regulatory subunit interact with positive charges proximal to phosphorylated residues to mediate site-specific dephosphorylation.

Reversible protein phosphorylation is an essential regulatory mechanism in eukaryotes (*1*). As one of four major groups of serine/threonine phosphatases, protein phosphatase 2A (PP2A)[1] holoenzymes are critically involved in a broad range of cellular processes, including cell-cycle progression, cytoskeletal dynamics, and growth factor signaling (*2*, *3*). PP2A exists as several multiprotein complexes. The catalytic (C) subunit can associate with a scaffolding (A) subunit to form dimeric PP2A. The AC dimer in turn recruits diverse regulatory B subunits to assemble the major form of PP2A, the ABC heterotrimer. PP2A regulatory subunits are encoded by three gene families, B (PR55), B′ (B56), and B″, the first two of which are highly conserved among eukaryotes. Alternative splicing of several regulatory subunits adds further complexity, such that any given mammalian cell type is thought to express dozens of distinct PP2A heterotrimers. Regulatory subunits exhibit diverse developmental and tissue expression profiles, and several have been shown to recruit PP2A to specific places in the cell (*4*−*6*). Recent crystal structures of PP2A heterotrimers containing B and B′ regulatory subunits have offered fascinating insights into how this master regulator of cellular signaling may operate. For instance, the Bα/PR55α regulatory subunit facilitates dephosphorylation of the microtubule-associated protein tau via low-affinity interactions of the top of the Bα β-propeller fold and the microtubule-binding repeats of tau (*7*). Structurally distinct, B′/B56 regulatory subunits feature an α-helical repeat architecture similar to that of the PP2A/A subunit (*8*, *9*). Despite this wealth of structural information, the molecular basis of PP2A substrate specificity remains incompletely defined.

We have previously reported that tyrosine hydroxylase (TH) is selectively dephosphorylated and inactivated by the neuronal PP2A/B′β holoenzyme (*10*). TH catalyzes the rate-limiting reaction in catecholamine biosynthesis, and a reduction in TH activity and dopamine levels is responsible for Parkinson's disease, the second most common neurodegenerative disorder. Multisite phosphorylation of the TH regulatory domain by several kinases [CaM kinase II → Ser19; extracellular signal-regulated kinase, ERK, and cyclin-dependent kinase 5 → Ser31; protein kinase A (PKA) → Ser40] can increase TH activity several-fold by relieving catecholamine end product inhibition (*11*, *12*). B′β localizes predominantly to the soma and dendritic compartment of dopaminergic neurons, in contrast to TH and the other catecholamine biosynthetic enzymes, which are additionally found at presynaptic terminals, where most catecholamine release occurs. Since low levels of PP2A/B′β in axons and terminals correlates with a high level of TH phosphorylation, PP2A/B′β may function to curb ectopic dopamine synthesis in the cell body and dendrite (*10*).

In this report, we investigate the sequence requirements for specific dephosphorylation of TH by PP2A/B′β. Guided by the PP2A/B′γ holoenzyme structure, we identify a conserved Glu

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*To whom correspondence should be addressed: Department of Pharmacology, University of Iowa Carver College of Medicine, 2-432 BSB, 51 Newton Rd., Iowa City, IA 52242. E-mail: stefan-strack@uiowa.edu. Phone: (319) 384-4439. Fax: (319) 335-8930.

[1]Abbreviations: ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; NGF, nerve growth factor; PKA, protein kinase A; PP, protein phosphatase; PP2A, protein phosphatase 2A; SE, standard error; TH, tyrosine hydroxylase.

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(Glu153 in B′β) that recognizes arginines in the TH N-terminus to mediate dephosphorylation of Ser19, -31, and -40. Studies on the regulation of NGF signaling by PP2A/B′β as well as phosphoproteomic analysis suggest that interaction of Glu153 with basic residues adjacent to phosphorylated residues constitutes a general mechanism of substrate recognition by the B′ family of PP2A subunits.

EXPERIMENTAL PROCEDURES

Mutagenesis. Deletion mutagenesis of rat B′β was conducted by polymerase chain reaction (PCR) with primers annealing to the N- and C-terminal borders of the truncated cDNA, with N-terminal primers also encoding a FLAG epitope tag. PCR fragments were ligated into pcDNA5/TO (BD Biosciences) via HindIII and NotI sites. Site-directed mutagenesis of FLAG-B′β was performed according to the QuikChange protocol (Invitrogen, Carlsbad, CA). Most plasmids expressing glutathione *S*-transferase (GST) fusions of TH and mitofusin 2 (Mfn2) were constructed by PCR amplification of TH/Mfn2 fragments and ligation into pGEX-2T restricted with BamHI and EcoRI. As templates for PCR, pGEX-2T-TH_{31-164} was provided by L. Moy and L.-H. Tsai (*13*) while full-length Mfn2 expression vectors were a gift of A. Santel and M. Fuller (*14*). We generated plasmids expressing GST−TH_{31-42} and GST−$Mfn2_{433-444}$ fusion proteins by phosphorylating double-stranded oligonucleotides and ligating them into pGEX-2T restricted with BamHI and EcoRI. GST expression plasmids were transformed into BL21 competent *Escherichia coli* cells, and GST fusion proteins were induced and purified by standard techniques.

Cell Lines and Culture. Tetracycline-inducible (T-Rex system, BD Biosciences) PC12 cell lines stably expressing FLAG epitope-tagged PP2A regulatory subunit mutants (EE152RR, E153R, and E152R) were generated as described previously (*15*). Inducible cell lines were cultured (37 °C and 5% CO_2) on collagen-coated plates in RPMI 1640 medium with 10% horse serum and 5% fetal bovine serum containing 2 μg/mL blasticidin and 200 μg/mL hygromycin. B′β wild-type and mutant expression was induced by the addition of doxycycline (1 μg/mL) for 2−3 days prior to analysis. Control cultures received 0.1% ethanol vehicle instead. COS-M6 cells used for transient transfections were cultured in DMEM supplemented with 10% fetal calf serum at 37 °C and 5% CO_2.

Biochemicals. The following reagents were obtained commercially: mouse TH antibody (Immunostar, Hudson, WI), rabbit phospho-Ser40 TH and phospho-(Thr/Tyr) ERK1/2 antibodies (Cell Signaling Technologies, Beverly, CA), phospho-Ser31 TH antibody (Chemicon, Temecula, CA), ERK1/2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), PP2A/C subunit antibody (BD Transduction Laboratories), L-[1-^{14}C]tyrosine (Moravek Biochemicals, Brea, CA), and rat nerve growth factor (NGF, 2.5S) (Upstate Biotechnology, Lake Placid, NY). Rabbit polyclonal antibodies recognizing total TH and Ser19-phosphorylated TH were provided by J. Haycock (*16*). The PP2A/B′β antibody was described previously (*10*).

Preparation of Phosphatase Substrates. GST−TH fusion protein, GST−Mfn2 fusion protein, and myelin basic protein (Sigma, St. Louis, MO) (3 μg/μL) were phosphorylated with the bovine heart PKA catalytic subunit [0.5 unit/μL (Sigma)] or recombinant ERK2 [5 ng/μL (Millipore/Upstate Biotech)] for 1 h at 30 °C in buffer containing 300 μM ATP, 50 μCi of [γ-^{32}P]ATP, 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EGTA, and 0.01% Triton X-100. Phosphorylation reactions were stopped by addition of 25 mM EDTA and desalting using spin columns (Micro Bio-Spin 6, Bio-Rad, Hercules, CA). ^{32}P-labeled substrates were stored aliquoted at −70 °C for up to 2 weeks.

Immunoisolation of PP2A and Phosphatase Assays. PP2A holoenzymes containing FLAG-tagged B′β wild type, EE152RR, and E153R were immunoprecipitated from stable PC12 cell lines via FLAG antibody agarose conjugates (M2, Sigma) as described previously (*10*). Aliquots of immunoprecipitates were solubilized in SDS sample buffer for immunoblot analyses with B′β and PP2A/C antibodies. For PP2A activity assays, immunoprecipitates were stored at −70 °C in 50% glycerol, 10% ethylene glycol, 20 mM Tris (pH 7.5), 5 mM dithiothreitol, 2 mM EDTA, and 0.1% Triton X-100. ^{32}P-labeled substrates were diluted in 2 mg/mL bovine serum albumin, 50 mM Tris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 1 mM benzamidine, and 1 mg/mL leupeptin. Phosphatase reactions were started by the addition of 2−5 μL of PP2A immunoprecipitates to 20 μL of diluted substrate, the mixtures incubated for 30 min at 30 °C with intermittent agitation on an Eppendorf shaking incubator, and the reactions terminated by the addition of TCA to a final concentration of 20%. After centrifugation at 22000*g*, acid-soluble ^{32}P was quantified by liquid scintillation counting. Reactions with varying enzyme concentrations established the linear range of the assay (< 20% of total ^{32}P released).

For the peptide dephosphorylation assays, wild-type and RR37AA mutant TH peptides (RFIGRRQ[pS]LIED and RFIGAAQ[pS]LIED) were synthesized commercially (GenScript, Piscataway, NJ). Phosphopeptide-directed PP2A activity was assayed using a colorimetric, malachite green-based kit according to the manufacturer's instructions (Serine/Threonine Phosphatase Assay System, Promega, Madison, WI). FLAG-immunoprecipitated PP2A/B′β holoenzymes were incubated with 100 μM phosphopeptide in buffer containing 50 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.1 mg/mL BSA, and 0.3% polyvinylidene pyrrolidone (PVP) 40 (50 μL reaction volume). After 30 min at 30 °C with intermittent shaking, 50 μL of dye reagent was added, and the reaction was allowed to continue for 60 min. Formation of the phosphomolybdate−malachite green complex was monitored by the absorbance at 600 nm, and phosphate concentrations were determined with reference to a standard curve.

Quantitative Immunoblotting and TH Activity Assays. TH and ERK phosphorylation in total PC12 cell lysates was quantified by immunoblotting with phosphorylation state-specific antibodies as described previously (*10*), except that an infrared scanner (Li-Cor Odyssey) was used to detect secondary antibodies. Detection linearity was established by probing varying amounts of lysate. An in situ dopamine biosynthesis assay, which monitors $^{14}CO_2$ produced from the conversion of L-[1-^{14}C]tyrosine to dopamine, was conducted as described previously (*10*).

Two-Dimensional Gel Electrophoresis and Phosphoprotein Staining. Inducible PC12 cells expressing either wild-type or E153R mutant B′β were incubated for 36−42 h with 1 μg/mL doxycycline or 0.1% EtOH vehicle, followed by lysis in a hypotonic solution [0.5% Triton X-100, 0.5 mM EDTA, 0.5% ampholytes (pH 3−10), 1 mM phenylmethanesulfonyl fluoride, 5 μg/mL leupeptin, and 1 μM microcystin LR]. Lysates were fractionated by centrifugation (10 min at 20000*g* and 4 °C), and supernatants were vacuum-concentrated 2−3-fold prior to

addition of 2 volumes of isoelectric focusing (IEF) sample buffer {7 M urea, 2 M thiourea, 1.25% 3-[(3-chloromidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 32 mM dithiothreitol, 2.5 mM tris(2-carboxyethyl)phosphine (TCEP), 0.5% amidosulfobetain-14 (ASB-14), 0.5% Triton X-100, 0.5% Zwittergent 3−10, and 0.8% ampholytes (pH 3−10)}. Immobilized pH gradient strips (11 cm, pH 5−8, Bio-Rad) were equilibrated with 225 μL of lysate (500−800 μg of protein) for 16−20 h, followed by IEF for 20000 V·h. After SDS−PAGE, gels were subjected to phosphoprotein staining with Pro Q Diamond (Invitrogen) according to the manufacturer's instructions and imaged with a Fuji FLA-7000 laser scanner. Gels were subsequently stained for total protein with Coomassie Blue R250 and imaged using a Li-Cor Odyssey infrared laser scanner (700 nm filter). Images of phosphoprotein- and total protein-stained gels of samples with or without doxycycline treatment were brought into registration using the ImageJ plug-in bUnwarpJ (*17*).

RESULTS

The Conserved Central Domain of B′β Mediates PP2A Holoenzyme Assembly and TH Dephosphorylation. The five mammalian isoforms (α−ε) of the B′/B56 family of PP2A regulatory subunits feature a highly conserved (~80% identical) central α-helical repeat domain, the structure of which has been determined as part of the PP2A/B′γ heterotrimer (*8*, *9*). The divergent N- and C-terminal regions may be largely unstructured and have been implicated in subcellular targeting (*18*, *19*). In an effort to determine the regions necessary for recruiting PP2A/B′β to dephosphorylate TH, we constructed a panel of N- and C-terminal B′β truncation mutants (Figure 1A). In initial experiments, these mutants were transiently expressed in COS cells and immunoprecipitated via an N-terminal FLAG tag to test for complex formation with endogenous PP2A scaffolding and catalytic subunits. The first 65 residues and the last 23 residues (475−497) were found to be dispensable for incorporation of B′β into the PP2A holoenzyme, while further N- or C-terminal truncation resulted in a loss of endogenous A and C subunits from the FLAG-B′β immunoprecipitate (Figure 1A,B). The region necessary for holoenzyme incorporation of B′β (residues 66−497) encompasses all tandem α-helical repeats but extends both N- and C-terminally beyond residues involved in direct interactions between B′γ and the A and C subunits (*8*, *9*), indicating that $B'\beta_{66-497}$ constitutes a structural unit. To determine whether the N-terminus of B′β is involved in TH regulation, we transiently overexpressed full-length B′β and the Δ1−30 and Δ1−65 truncation mutants to similar levels in PC12 cells, a catecholamine-secreting cell line that expresses high levels of TH. The 65 N-terminal amino acids of B′β are not required for targeting PP2A to TH, since all three constructs promoted TH dephosphorylation to similar levels [ca. 70% phospho-Ser40 TH immunoreactivity compared to empty vector-transfected cells (Figure 1C,D)]. Similar experiments in which B′β truncated at the C-terminus was overexpressed indicated that, likewise, residues 475−497 are expendable for dephosphorylation of TH in intact cells (data not shown).

Glu153 of PP2A/B′β Is Important for TH Dephosphorylation and Inactivation in Intact Cells. With the data described above pointing to the conserved central domain of B′β as being important for TH modulation, we turned to the crystal structure of the PP2A/B′γ heterotrimer as a guide to identify B′β residues involved in substrate recognition. The

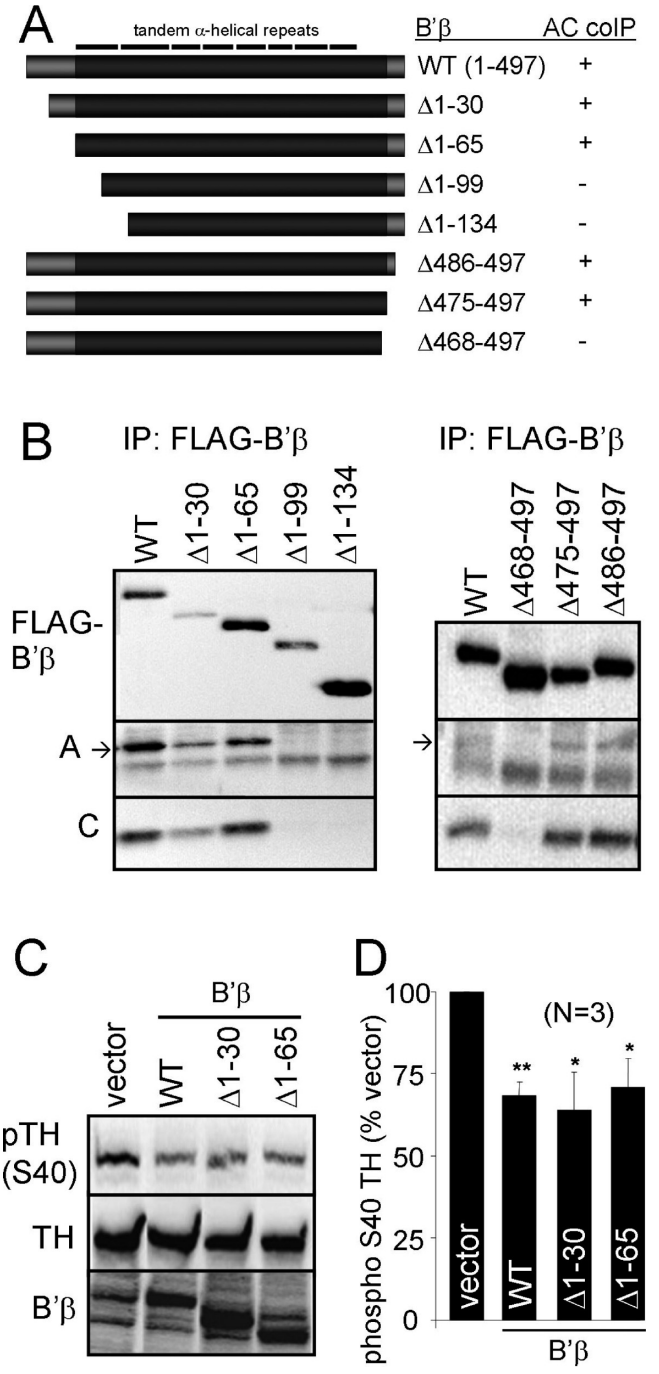

FIGURE 1: The divergent N-terminus of B′β is dispensable for TH dephosphorylation. (A) Domain diagram of B′β truncation mutants and summary of PP2A heterotrimer incorporation experiments. Light gray areas are not required for A and C subunit association. (B) The indicated FLAG-tagged B′β constructs (WT, wild-type) were transiently expressed in COS cells and assessed for holoenzyme incorporation by co-immunoprecipitation (IP) of FLAG-B′β with endogenous A (arrow) and C subunits. (C and D) Total lysates of PC12 cells transiently transfected with the indicated plasmids were analyzed for TH phosphorylation at Ser40 (pTH S40), total TH, and B′β expression. Shown are a representative blot (C) and a quantification of phospho-Ser40 TH over total TH relative to empty vector-transfected cells (D, mean ± SE from three experiments). By a Student's *t* test compared to vector control, one asterisk indicates $p < 0.05$ and two asterisks indicate $p < 0.01$.

concave surface of B′γ (and by extension other B′ regulatory subunits) is oriented toward the catalytic subunit and has a high negative charge density (Figure 2A), which was postulated to

recruit positively charged phosphoproteins via electrostatic mechanisms (*8*, *9*). We focused on two adjacent glutamic acid residues, because of their near invariance in B′ (but not B or B″) subunits from yeast to humans and their proximity to the catalytic cleft on the C subunit (Figure 2A,B). Glu152 and Glu153 (B′β numbering) are located at the tip of an extended loop within tandem α-helical repeat 2, which makes several contacts with the C subunit. Glu152 does not engage the C subunit directly, and its exposed side chain is compatible with substrate interactions. The carbonyl oxygen of Glu153, on the other hand, forms a hydrogen bond with Arg268 of the C subunit, while its carboxyl group forms van der Waals contacts with microcystin-LR, an active site inhibitor that was cocrystallized with the PP2A/B′γ heterotrimer [Figure 2A (*8*, *9*)].

To explore the role of Glu152 and Glu153 in PP2A/B′β substrate specificity, we generated a panel of B′β single and double mutants with charge-neutralizing (Glu → Gln) and charge-reversing (Glu → Arg) substitutions [B′β E152Q, E152Q/E153Q (denoted EE152QQ), E153Q, E152R, E153R, and E152R/E153R (denoted EE152RR)]. Transient transfection and co-immunoprecipitation experiments in COS cells demonstrated that none of these mutations impaired association of FLAG-B′β with the endogenous PP2A catalytic subunit (Figure 2C). Providing a negative control, phosphomimetic (Ser → Asp) mutations of a predicted and an established (*20*) phosphorylation site at an intersubunit interface (SS367DD) abolished incorporation of B′β into the PP2A heterotrimer (Figure 2C).

To test the effect of Glu152 and/or Glu153 mutant B′β on TH phosphorylation and catecholamine synthesis, we generated clonal PC12 cells that express FLAG-B′β from a tetracycline/doxycycline (Dox)-inducible promoter (*10*). As with transient expression in COS cells, wild-type B′β and the three charge-reversal mutants (E152R, E153R, and EE152RR) were inducibly expressed to similar levels and associated with similar amounts of PP2A/C in PC12 cells (Figures 2D and 3A and insets of Figure 4B,D). As previously reported (*10*), wild-type B′β overexpression promoted dephosphorylation of TH at Ser19, Ser31, and Ser40 (Figure 3A,B). Compared to sister cultures treated with vehicle, Dox-induced TH dephosphorylation ranged from ~20% (Ser19) to >50% (Ser40). Mutation of Glu152 to Arg (E152R) did not compromise the ability of B′β to dephosphorylate TH, arguing against a critical role for this residue in substrate specificity. In contrast, inducible expression of the two mutants with substitutions at Glu153 (E153R and EE152RR) failed to dephosphorylate TH at Ser19, Ser31, or Ser40 (Figure 3A,B). In situ TH activity assays were conducted to determine whether Glu153 is also important for modulation of dopamine synthesis by PP2A/B′β (*10*). The amount of $^{14}CO_2$ released from PC12 cells cultured in the presence of L-[1-^{14}C]-tyrosine is a measure of TH activity, since TH is rate-limiting in the two-step conversion of tyrosine to dopamine (Figure 3C). Inducible overexpression of wild-type B′β resulted in a robust (>50%) inhibition of $^{14}CO_2$ release, paralleling effects on TH phosphorylation. In comparison, cell lines expressing the two Glu153 mutants were significantly compromised in their ability to downregulate TH activity, even though B′β E153R and EE152RR induction still attenuated dopamine synthesis compared to parallel cultures that had not received doxycycline [30–40% (Figure 3D)], and compared to induction of an unrelated PP2A regulatory subunit (*10*). The partial effect of Glu153 charge reversal on TH activity in conjunction with its fully penetrant

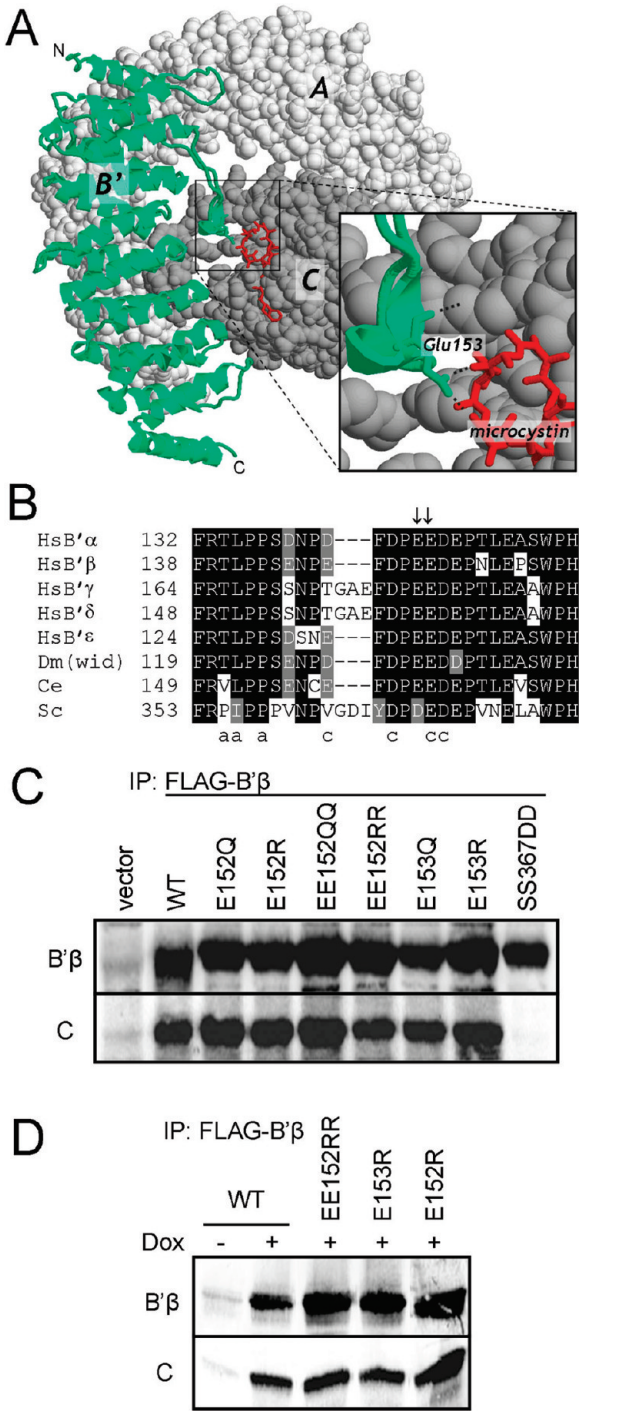

FIGURE 2: Glu152 and Glu153 mutations do not affect incorporation of B′β into the PP2A heterotrimer. (A) Structure of the PP2A/B′ heterotrimer (A and C subunits in space filling and B′ in ribbon representation) highlighting Glu153 (B′β numbering) at the tip of a loop that connects to the active site (inset). The carboxyl group of Glu153 contacts microcystin, while its carbonyl oxygen contacts Arg268 on the C subunit (stippled lines). (B) Phylogenetic alignment of loop residues surrounding Glu152 and Glu153 in B′β (Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; wid, widerborst; Ce, *Caenorhabditis elegans*; Sc, *Saccharomyces cerevisiae*; a, A subunit contacts; c, C subunit contacts). (C) The indicated plasmids were transiently expressed in COS cells, and lysates were analyzed for co-immunoprecipitation of FLAG-B′β with the endogenous PP2A catalytic (C) subunit. (D) Stable PC12 cell lines were treated for 2 days with either vehicle (−) or doxycycline (Dox) to induce expression of wild-type or mutant FLAG-B′β. FLAG immunoprecipitates probed for B′β and the C subunits demonstrate equivalent PP2A holoenzyme association of wild-type and mutant B′β.

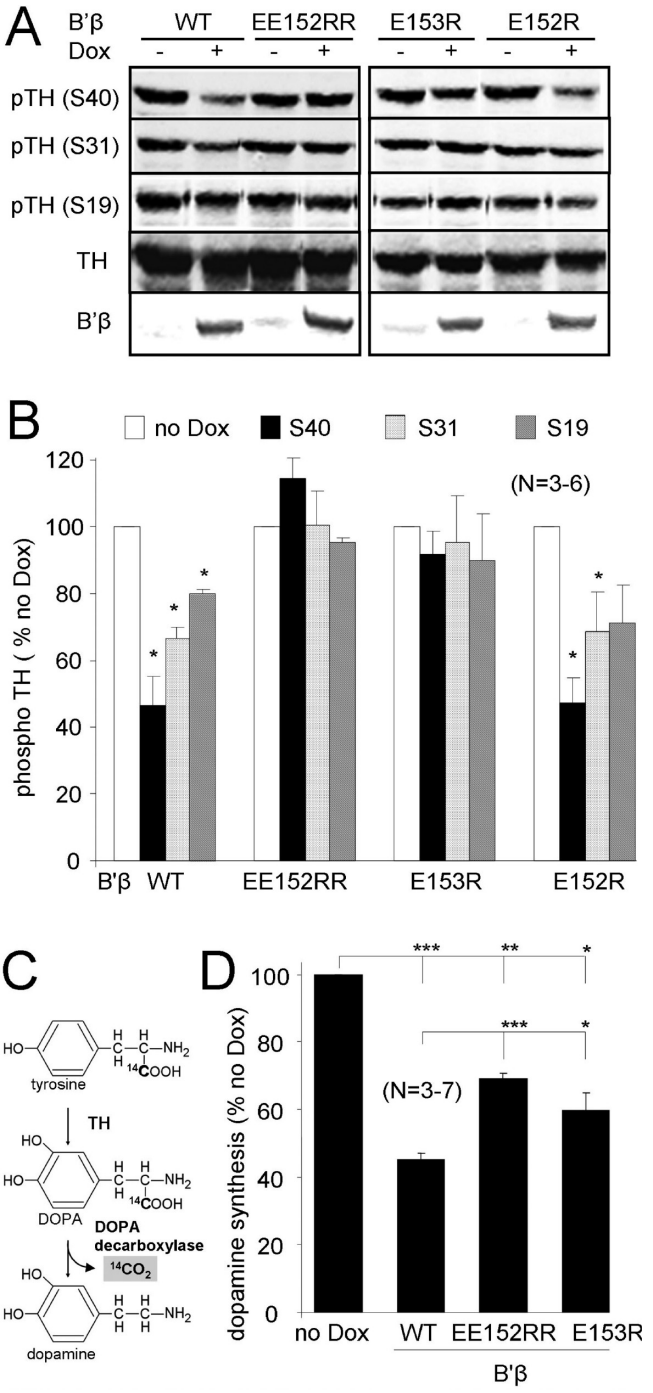

FIGURE 3: B′β Glu153 is critical for TH dephosphorylation and inactivation in PC12 cells. (A and B) PC12 cell lines were treated with or without doxycycline (Dox) to induce wild-type or mutant B′β expression. Total cell lysates were then analyzed for TH phosphorylation with the indicated antibodies. Shown are representative blots (A) and quantification of phospho (p)TH over TH signals normalized to uninduced (no Dox) cultures (mean ± SE of three to six independent experiments). (C and D) Stable PC12 cell lines were induced with or without Dox and incubated with L-[1-^{14}C]tyrosine, and $^{14}CO_2$ release was assessed by scintillation counting as a measure of dopamine synthesis. Panel C depicts the assay principle, and panel D shows TH activity relative to uninduced (no Dox) controls (mean ± SE of three to seven experiments). One asterisk indicates $p < 0.05$. Two asterisks indicate $p < 5 \times 10^{-4}$. Three asterisks indicate $p < 5 \times 10^{-5}$.

effect on TH phosphorylation indicates that PP2A/B′β regulates TH activity by N-terminal Serine phosphorylation-dependent and -independent mechanisms, with only the former requiring B′β Glu153.

B′β Glu153 Is an in Vitro Specificity Determinant. We previously showed that PP2A/B′β directly and specifically dephosphorylates the in vitro PKA-phosphorylated regulatory domain of TH (residues 31−164) (*10*). To examine the influence of Glu153 on PP2A/B′β activity in vitro, we immunoisolated the PP2A holoenzyme containing wild-type or mutant FLAG-B′β from Dox-induced PC12 cells and conducted phosphatase assays with Ser40-phosphorylated GST−TH_{31-164} fusion protein. As in intact cells, PP2A/B′β EE152RR displayed an ∼50% reduction in TH-directed phosphatase activity. However, the mutant performed as well as wild-type B′β when assayed for dephosphorylation of two other substrates, PKA-phosphorylated myelin basic protein and a GST−mitofusin 2 fusion protein ($Mfn2_{421-488}$) (Figure 4B and data not shown), the latter of which contains a single in vitro PKA phosphorylation site at Ser442 (*21*). Figure 4C shows average TH/Mfn2 dephosphorylation ratios for wild-type and EE152RR B′β as a measure of specificity. We next assayed charge-neutralizing (glutamine) and charge-reversing (arginine) substitutions of B′β Glu153 for effects on TH-directed phosphatase activity. Either mutation inhibited Ser40-TH dephosphorylation by ∼50% (Figure 4D), indicating it is the lack of a negative charge at this position that underlies the activity deficit. We thus conclude that PP2A/B′β Glu153 is required for efficient dephosphorylation of TH in vitro and in intact cells, but not for in vitro dephosphorylation of two other substrates.

It is worth noting that the observed effects of B′β Glu153 substitution on TH dephosphorylation were more dramatic in intact cells than in vitro. We attribute the comparatively modest decrease in TH-directed PP2A/B′β activity after replacement of Glu153 to a partial dissociation of PP2A heterotrimers into highly active AC dimers or catalytic subunits during the phosphatase assay. Indeed, addition of a macromolecular crowding agent, polyvinylpyrrolidone [M_r = 40000 (PVP-40, 0.3%)], to stabilize the PP2A holoenzyme was found to lower overall in vitro phosphatase activity but also to accentuate the activity difference between wild-type and Glu153 mutant B′β (not shown); 50% is therefore a lower estimate of the contribution of Glu153 to PP2A/B′β activity.

TH Arg37 and Arg38 Mediate Dephosphorylation by PP2A/B′β. To identify phosphatase selectivity determinants in the TH regulatory domain, progressive C-terminal truncations were expressed as GST fusion proteins and ^{32}P-phosphorylated by PKA at Ser40 (Figure 4A). Phosphatase assays with wild-type and E153R mutant PP2A/B′β demonstrated that TH dephosphorylation was independent of residues C-terminal of the PKA phosphorylation site; i.e., TH_{31-42} was dephosphorylated as well as TH_{31-164} with charge reversal at Glu153 decreasing the specific activity of PP2A/B′β toward both substrates by approximately half (Figure 4E,F).

Having implicated residues vicinal to the Ser40 phosphorylation site in TH as possible recognition sites for B′β Glu153, we next performed phosphatase assays with various Mfn2 fragments that include the PKA phosphorylation site, Ser442 (Figure 5A). Intriguingly, while dephosphorylation of the longest GST fusion protein ($Mfn2_{421-488}$) was independent of Glu153 (wild-type B′β/E153R B′β activity ratio of ∼1), C-terminal truncation of this region progressively favored dephosphorylation by wild-type PP2A/B′β. Similar to TH, the shortest Mfn2 fragment encompassing Ser442 ($Mfn2_{433-444}$) was dephosphorylated by PP2A/B′β E153R with an efficiency approximately half that of the wild-type phosphatase (Figure 5B,C). Because the only residues in

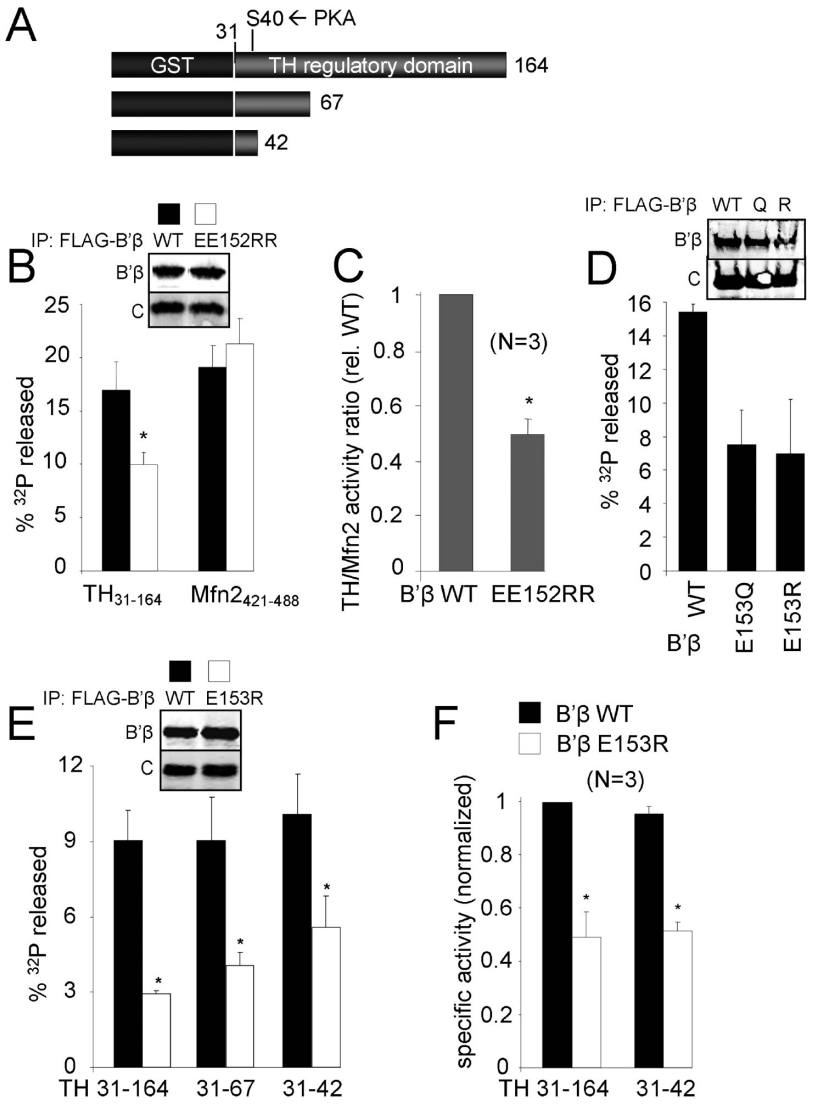

FIGURE 4: B′β Glu153 recognizes selectivity determinants close to the TH Ser40 phosphorylation site. (A) Diagram of glutathione *S*-transferase (GST) TH fusion proteins used in this figure. (B−F) Wild-type and mutant B′β-containing PP2A holoenzymes were isolated via FLAG immunoprecipitation (IP) from stable PC12 cell lines (B, C, E, and F) or from transiently transfected COS-1 cells (D). Enzyme aliquots containing equivalent C subunit (insets of panels B, D, and E) were assayed toward the indicated ^{32}P-labeled GST fusion proteins (in vitro phosphorylated by PKA on TH Ser40 and Mfn2 Ser442). Representative phosphatase assays are shown in panels B, D, and E (mean ± standard deviation of triplicate reactions), while data summaries are shown in panels C and F (mean ± SE of three assays). An asterisk indicates $p < 0.05$.

common between TH_{31-42} and $Mfn_{433-444}$ are the phosphorylated serine and the two arginines at the −2 and −3 positions (Figure 5A), these results point to residues that comprise the PKA consensus phosphorylation sequence (RRX[S/T]) as determinants for PP2A/B′β-mediated dephosphorylation as well. With respect to why dephosphorylation of longer Mfn2 fragments or myelin basic protein does not depend on Glu153, we surmise that basic residues distant from the phosphorylation site (C-terminal in the case of Mfn2) engage in charge−charge interactions with acidic residues on the concave surface of the B′ regulatory subunit, thereby overriding the requirement for the active site proximal Glu153.

To directly address the role of basic amino acids in PP2A/B′β substrates, Arg37 and Arg38 were mutated to Ala, initially in the context of a GST−TH fusion protein (GST−TH_{26-41}). Since the RR37AA mutation abrogates the PKA consensus, wild-type and mutant TH fusion proteins were in vitro phosphorylated with ERK2 at Ser31 for subsequent phosphatase assays (Figure 6A). Consistent with results obtained in intact cells (Figure 3B), Ser31-phosphorylated TH was a better substrate for wild-type than for E153R mutant PP2A/B′β. In contrast, dephosphorylation of ERK-phosphorylated myelin basic protein was not significantly affected by the B′β mutation. Neutralization of TH Arg37 and Arg38 decreased the level of dephosphorylation of Ser31 by ~50% and eliminated the difference in specific activity between wild-type and Glu153 mutant B′β (Figure 6B,C). To confirm the importance of Arg37 and Arg38 for dephosphorylation of the PKA site, Ser40-phosphorylated TH peptides were synthesized [wild-type and RR37AA TH_{31-42} (Figure 6A)] and used in malachite green-based phosphatase assays. Arg → Ala substitution of phospho-Ser40 TH_{31-42} inhibited dephosphorylation by wild-type PP2A/B′β (~50%) but had no influence on dephosphorylation by Glu153 mutant PP2A/B′β (Figure 6D). In conclusion, these TH Ser31 and Ser40 dephosphorylation experiments support a model of PP2A substrate recognition, in which B′β Glu153 directs PP2A to phosphorylated residues with proximal arginines on either side.

Glu153 Is Critical for Positive Regulation of NGF Signaling by PP2A/B′β. Glu153 is perfectly conserved in B′ regulatory subunits across the eukaryotic lineage (Figure 2B),

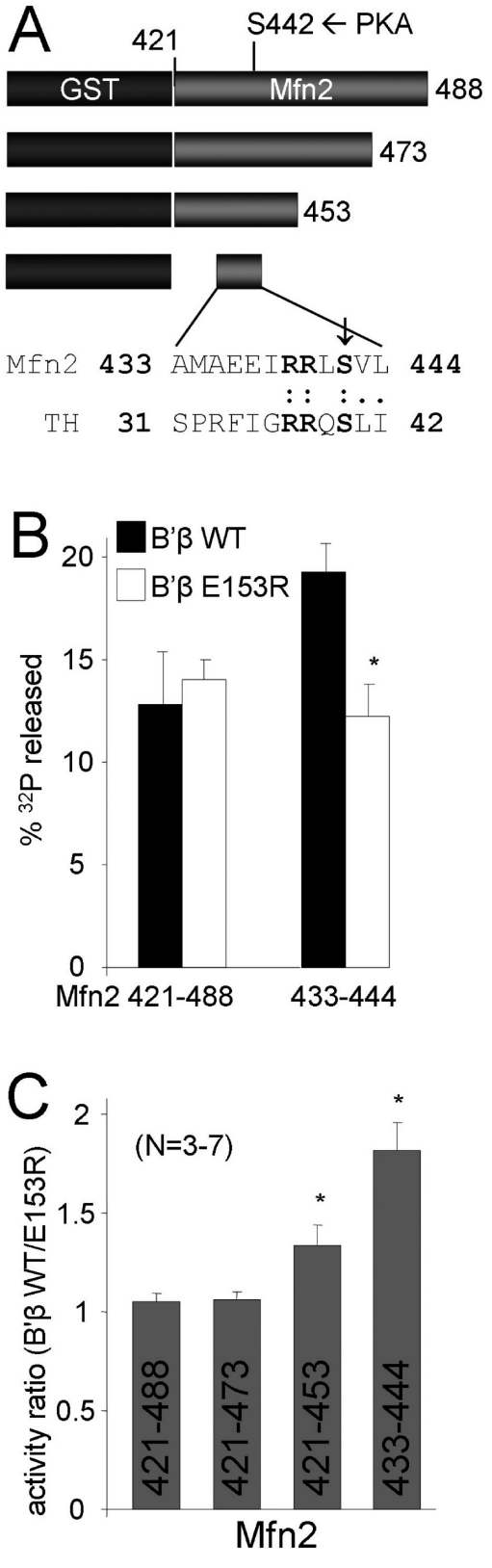

FIGURE 5: Truncation of Mfn2 to the PKA phosphorylation site renders dephosphorylation dependent on Glu153 of B′β. (A) Diagram of GST−Mfn2 fusion proteins used in this figure and alignment of the PKA phosphorylation sites (arrow) in Mfn2 and TH. (B and C) PKA-phosphorylated Mfn2 fusion proteins were assayed for dephosphorylation by wild-type or mutant PP2A/B′β. A representative assay is shown in panel B (mean ± standard deviation of triplicate experiments) and a summary in panel C (ratio of wild-type B′β and E153R B′β activities; mean ± SE of three to seven assays). An asterisk indicates $p < 0.05$.

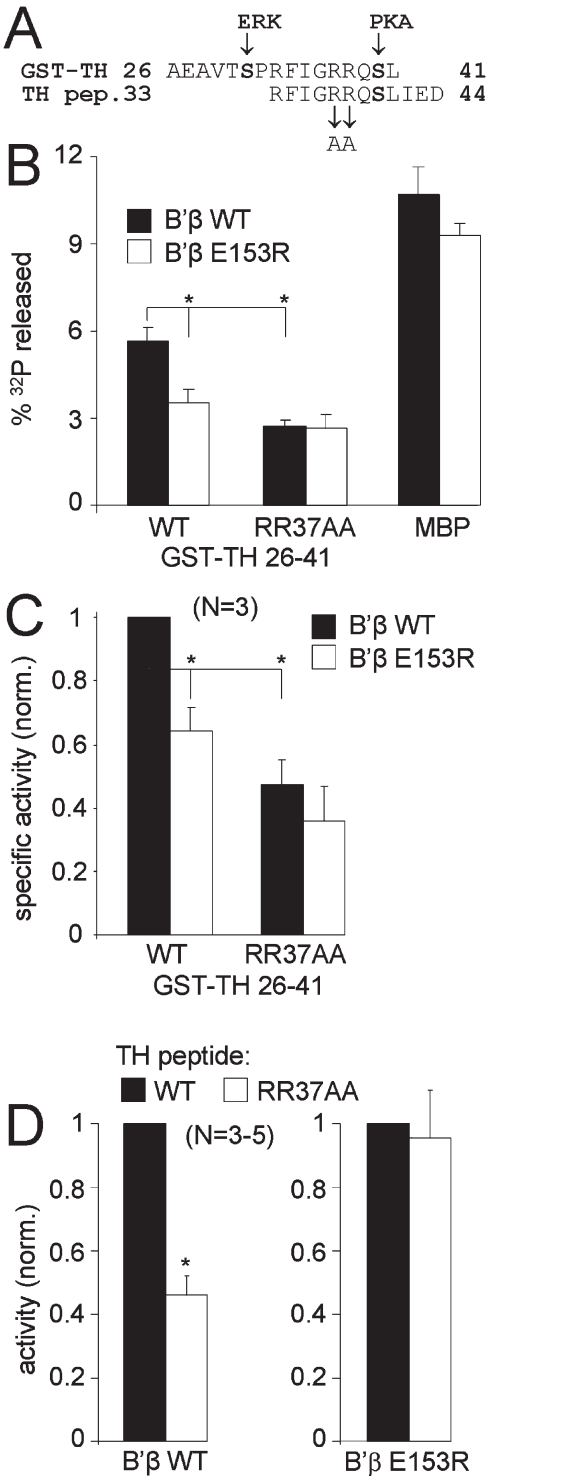

FIGURE 6: Arg37 and Arg38 are required for efficient dephosphorylation of TH by wild-type but not Glu153 mutant PP2A/B′β. (A) Sequence of the wild-type and Arg37 and Arg38 mutant TH−GST fusion protein and peptide, indicating ERK (Ser31) and PKA (Ser40) phosphorylation sites. (B and C) The indicated proteins were phosphorylated in vitro with ERK2 and assayed for dephosphorylation by wild-type and mutant PP2A/B′β: (B) representative assay showing the mean ± standard deviation of triplicate experiments and (C) summary of specific activities normalized to reactions with wild-type proteins (mean ± SE of three to five assays). (D) Phospho-Ser40 containing wild-type and Arg mutant TH peptides were assayed for dephosphorylation by wild-type and mutant PP2A/B′β using a colorimetric assay. Phosphatase activities are shown as means ± SE of three to five independent assays normalized to wild-type TH peptide. An asterisk indicates $p < 0.05$.

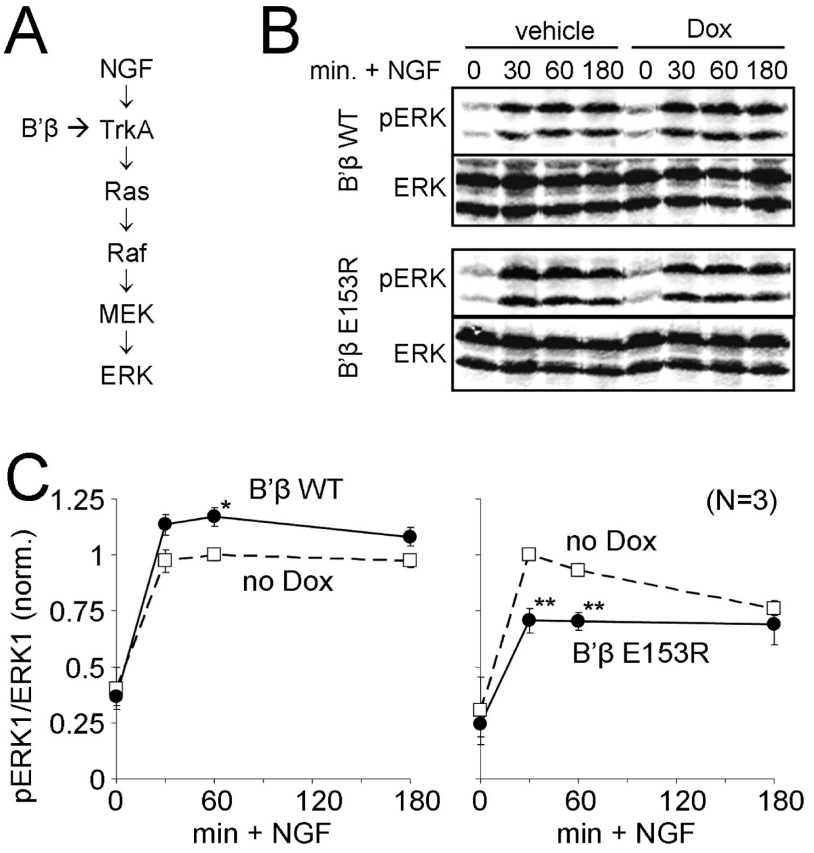

FIGURE 7: Glu153 is required for B′β-mediated stimulation of NGF signaling. (A) Schematic of NGF signal transduction to ERK. PP2A/B′β sustains NGF signaling by enhancing Tyr autophosphorylation of the NGF receptor TrkA (*22*). (B and C) PC12 cells inducibly expressing wild-type or E153R mutant B′β were cultured with or without Dox and stimulated with 20 ng/mL NGF for the indicated times, and total extracts were probed for phosphorylated (pERK) and total ERK. Blots from a representative experiment are shown in panel B, while panel C quantifies ERK phosphosphorylation from three experiments (mean ± SE of pERK1 divided by total ERK1 normalized to the highest value for vehicle-treated cells). One asterisk indicates $p < 0.05$; two asterisks indicate $p < 0.01$.

suggesting that this residue plays an important role in the dephosphorylation of many if not all substrates of this class of PP2A regulatory subunits. We previously showed that B′β and B′δ recruit PP2A to the neurotrophin receptor TrkA to sustain NGF-dependent tyrosine autophosphorylation of TrkA, signaling to ERK and Akt, and neuronal differentiation. Because the PP2A inhibition results in TrkA hyperphosphorylation at Ser/Thr residues, PP2A/B′β/δ may dephosphorylate inhibitory phosphorylation sites in the TrkA C-terminus to attenuate receptor desensitization (*22*). To investigate whether Glu153 participates in the augmentation of NGF responses by B′β, PC12 cells that inducibly overexpress either wild-type or E153R mutant B′β were stimulated with NGF for up to 3 h and ERK activation was quantified via phospho-specific antibodies. While wild-type B′β induction promoted a small but robust increase in the level of NGF-induced ERK phosphorylation, the Glu153 mutant actually inverted this effect, blunting ERK activation at all but the longest time point examined (Figure 7B,C). This apparent dominant-negative action contrasts with the inability of the B′β mutant to modulate TH phosphorylation in cells and could be explained by B′β E153R competing with endogenous B′ regulatory subunits for binding to TrkA. Irrespective of the precise mechanism by which PP2A/B′β stimulates neurotrophin signaling, our results point to a critical role for Glu153 in the B′β-mediated regulation of both TH and TrkA.

Glu153 Is Necessary for Dephosphorylation of Many Cellular B′β Substrates. Since Glu153 is not required for efficient dephosphorylation of MBP and longer GST−Mfn2 fusion proteins in vitro, we wondered if Glu153-independent B′β substrates might exist in cells. To address this question, PC12 cells that inducibly express wild-type B′β or the E153R mutant were analyzed by two-dimensional gel electrophoresis and staining with Pro Q Diamond, a fluorescent dye that detects phosphoproteins with nanogram sensitivity. To reduce spot complexity, proteins were first fractionated according to their solubility in hypotonic buffer containing 0.5% Triton X-100 and then analyzed by isoelectric focusing on narrow pH gradients (pH 5−8). Solubilization with nonionic detergent resulted in a roughly equal division of proteins into supernatant and particulate fractions, with only a few, high-abundance proteins detectable in both fractions (data not shown). While inducible expression of B′β was accompanied by only modest changes in the migration pattern of proteins in the Triton X-100 insoluble fraction, multiple putative B′β substrates were detected in the Triton X-100 soluble fraction. In the pH 5.5−6.5 and 50−100 kDa range of the gel, Pro Q Diamond labeled several trains of spots separated by ~0.1 pH unit, likely corresponding to variants of the same protein with numbers of incorporated phosphates increasing from the basic to the acidic end of the gradient (Figure 8A). Indicative of dephosphorylation, B′β induction with doxycycline resulted in reduced labeling of multiple spots in the pH 5.5−6.5 range (red circles) and increased labeling of a few spots at more basic pH (blue circles). We then compared cellular substrates of wild-type and Glu153 mutant B′β, staining two-dimensional (2D) gels of Triton X-100 soluble extracts sequentially with Pro Q Diamond for phosphoproteins and Coomassie

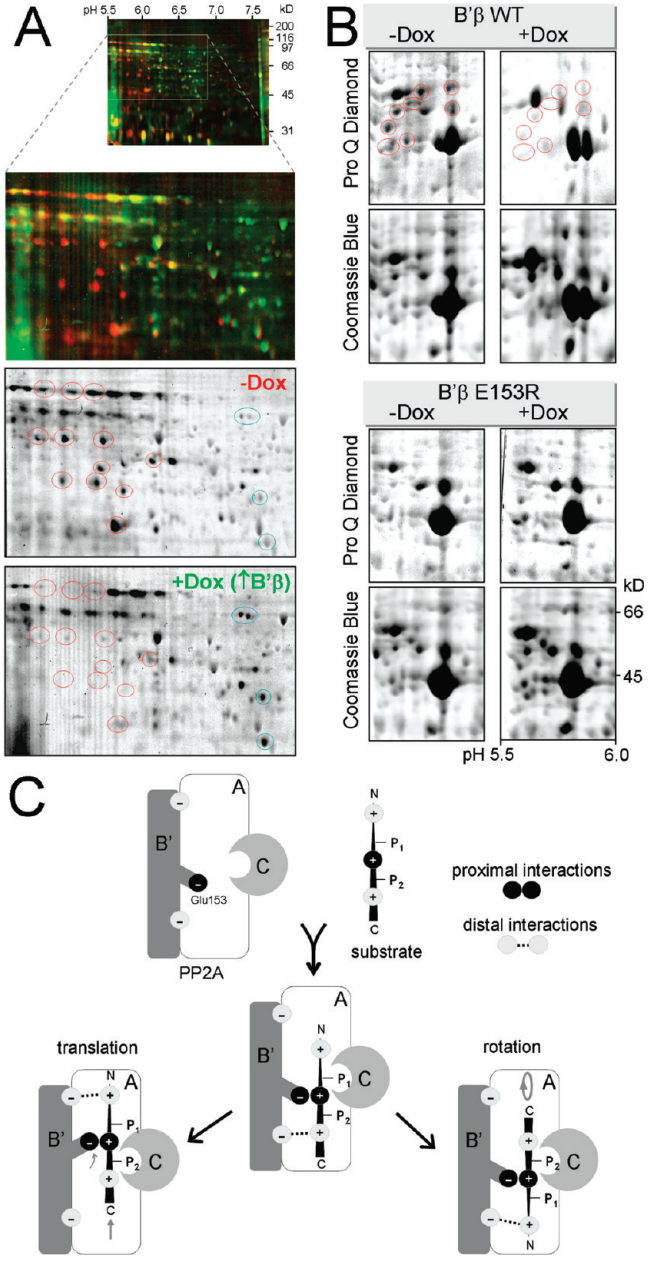

FIGURE 8: Glu153 is required for dephosphorylation of multiple B′β substrates in PC12 cells. Lysates of stable PC12 cell lines cultured for 36−42 h either in the absence (−) or in the presence (+) of doxycycline (Dox) to induce expression of wild-type (A and B) or E153R (B) PP2A/B′β were fractionated into Triton X-100 soluble and insoluble fractions. Soluble proteins were subjected to isoelectric focusing over pH 5.0 to 8.0 gradients, further separated by SDS−PAGE, and then stained with Pro Q Diamond to detect phosphoproteins. In panel B, the same gels were restained for total protein with Coomassie Blue R250. In panel A, gels from uninduced (−Dox) and B′β-expressing (+Dox) cells are shown individually and as a pseudocolored overlay subsequent to digital warping to bring spot patterns into registration. Red and blue circles highlight increases and decreases in spot intensity, respectively, upon Dox addition. Results are representative of four or more experiments. (C) Model of PP2A/B′β substrate interactions (see the text for details).

Blue for total proteins. Again, wild-type B′β induction promoted apparent dephosphorylation of multiple spots in the pH 5.5−6.0 range but did not appreciably alter the total protein pattern. Inducible expression of B′β E153R, on the other hand, had no reproducible effect on phosphoprotein staining in this molecular mass and pH range (Figure 8B). We did detect B′β-induced changes in phosphorylation that were independent of Glu153 in other regions of the 2D gel, but those changes affected fewer, isolated spots to a lesser degree (not shown). Global phosphoprotein analyses therefore indicate that Glu153 is important for dephosphorylation of the majority of B′β substrates in PC12 cells.

DISCUSSION

Recent findings challenge the notion of protein phosphatases as constitutively active, promiscuous enzymes that provide the backdrop for regulated signaling by protein kinases (for a review, see ref *3*). It is in this context that we provide here the first evidence that PP2A regulatory subunits recognize sequence determinants proximal to phosphorylation sites to effect substrate selection. Specifically, we found that a conserved Glu in the B′ family of PP2A regulatory subunits is critical for negative regulation of TH and positive regulation of NGF signaling through TrkA (*22*), as well as for dephosphorylation of multiple, as yet unidentified B′ substrates in cells. Glu153 mutant B′β cannot discriminate between arginine-containing and -lacking phosphopeptides, defining a loose dephosphorylation consensus for PP2A/B′ heterotrimers.

Protein kinase consensus sequences are generally directional; e.g., Arg37 and Arg38 direct PKA to phosphorylate Ser40, but not Ser31 in TH. PP2A/B′, on the other hand, utilizes Arg37 and Arg38 to dephosphorylate both Ser40 and Ser31 (and presumably Ser19). Two models can account for nondirectional site selection by Glu153 of PP2A/B′β (Figure 8C). In the rotation model, peptides with two phosphorylation sites flanking a group of positive charges can bind to a subunit-spanning substrate docking site in either orientation. In the translation model, conformational flexibility of the loop that extends Glu153 toward the catalytic center allows the dually phosphorylated peptide to slide between positions that align the two phosphates with the catalytic cleft. We favor the second model because the "substrate selectivity loop" appears relatively unconstrained by inter- and intrasubunit interactions and because conformational adaptability is well-documented by the available PP2A structures (for a review, see ref *23*).

X-ray crystal structures of both PP1 (*24*) and PP2A holoenzymes (*8*, *9*) revealed clusters of negatively charged residues on the regulatory subunit that are thought to attract positively charged substrates for dephosphorylation by the catalytic subunit. In the case of the PP2A holoenzyme containing Bα, an acidic groove on the top of the seven-blade β-propeller fold (> 20 Å from the catalytic site) was shown by site-directed mutagenesis to participate in dephosphorylation of tau. Because the positively charged microtubule binding repeats of tau interact weakly with Bα, a model was proposed in which sequential interactions of the Bα β-propeller with the tau microtubule binding repeats allow for efficient dephosphorylation of multiple sites along the tau primary sequence (*7*). Similar substrate interactions distal to the catalytic site were suggested to occur along the highly acidic, concave surface of the B′γ regulatory subunit, although this was not tested experimentally (*8*, *9*). Our results are compatible with distal interactions playing a role for some but not all PP2A/B′ substrates, because dephosphorylation of myelin basic protein and $Mfn2_{421-488}$ did not depend on Glu153, while dephosphorylation of shorter Mfn2 and all TH fragments was sensitive to Glu153 substitution.

In addition to substrate interactions with the conserved core domain of regulatory subunits, a subcellular targeting mechanism involving divergent domains clearly contributes to PP2A substrate selectivity. For instance, despite the conservation of Glu153, PP2A/B′γ overexpression is unable to modulate TH phosphorylation in PC12 cells, presumably because of a nuclear localization sequence in this B′ isoform's C-terminal tail (*6*). The striated muscle enriched B′α isoform associates via its C-terminus with ankyrin, which likely limits its substrates to cytoskeletal and membrane proteins (*7*). We thus propose a three-tiered mechanism for PP2A substrate recognition in which (1) isoform-specific subcellular targeting and anchoring sequences define a "sphere of influence", (2) electrostatic interactions involving conserved core residues distal from the active site increase the local concentration of oppositely charged substrates, and (3) residues in the proximity of the active site (e.g., Glu153 in B′β) restrict dephosphorylation to specific sites (Figure 8C).

Even though B and B′ family regulatory subunits share no sequence homology and adopt dissimilar folds (β-propeller and $\alpha-\alpha$ superhelix, respectively), one feature is conspicuously conserved between the PP2A/Bα and PP2A/B′γ heterotrimer structures, namely, an extended regulatory subunit loop that touches the catalytic site in the C subunit [Figure 2A (*7*−*9*)]. Like the Glu residue that is the focus of this report, the two residues that form the tip of the loop in B family subunits (Leu87 and Lys88 in Bα) are phylogenetically invariant. Whether these residues determine distinct site selection by B family regulatory subunits is an important question that will be the subject of future studies.

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