Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56δ subunit

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Our previous studies of DARPP-32 in striatal slices have shown that activation of D1 receptors leads to cAMP-dependent dephosphorylation of Thr-75, the Cdk5 site in DARPP-32. In the current study, we have elucidated a mechanism whereby protein phosphatase 2A (PP2A) is activated by a cAMP/PKA-dependent pathway, leading to dephosphorylation of Thr-75. PP2A consists of a catalytic C subunit that associates with the scaffolding A subunit and a variety of B subunits. We have found that the A/C subunits of PP2A, in association with the B56δ (or PPP2R5D) regulatory subunit, is an active DARPP-32 phosphatase. The B56δ subunit expressed in HEK293 cells forms a heterotrimeric assembly that catalyzes PKA-mediated dephosphorylation at Thr-75 in DARPP-32 (also cotransfected into HEK293 cells). The B56δ subunit is phosphorylated by PKA, and this increases the overall activity of PP2A *in vitro* and *in vivo*. Among four PKA-phosphorylation sites identified in B56δ *in vitro*, Ser-566 was found to be critical for the regulation of PP2A activity. Moreover, Ser-566 was phosphorylated by PKA in response to activation of D1 receptors in striatal slices. Based on these studies, we propose that the B56δ/A/C PP2A complex regulates the dephosphorylation of DARPP-32 at Thr-75, thereby helping coordinate the efficacy of dopaminergic neurotransmission in striatal neurons. Moreover, stimulation of protein phosphatase activity by this mechanism may represent an important signaling pathway regulated by cAMP in neurons and other types of cell.

cAMP | DARPP-32 | protein phosphorylation

DARPP-32 is a phosphoprotein that is highly enriched in dopaminoceptive medium-sized spiny neurons in the striatum and nucleus accumbens (1, 2). A variety of biochemical studies as well as targeted deletion and mutation of DARPP-32 in mice have shown that DARPP-32 plays a critical role in the actions of dopamine as well as in the actions of antipsychotic drugs, drugs of abuse, and other agents that modulate dopamine levels in the brain (2–5). Through activation of the D1 subclass of receptors, dopamine increases cAMP, activates protein kinase A (PKA), and phosphorylates Thr-34 of DARPP-32. Phosphorylation at Thr-34 converts DARPP-32 into a potent, high-affinity inhibitor of the broad specificity serine/threonine protein phosphatase, PP-1, leading to increased phosphorylation of many physiologically important substrates in medium spiny neurons, including neurotransmitter receptors, voltage-gated ion channels, ion pumps, protein kinases, and transcription factors (1, 2).

In addition to Thr-34, DARPP-32 is phosphorylated at multiple sites by several protein kinases, including CK1, CK2 and Cdk5 (6–9). In particular, phosphorylation of Thr-75 by Cdk5 blocks PKA-mediated phosphorylation of Thr-34 of DARPP-32, thereby modulating the efficacy of the dopamine/D1/cAMP/PKA/DARPP-32/PP1 signaling cascade (8). Our previous studies have found that there is a reciprocal relationship between the phosphorylation status of Thr-34 and Thr-75 (10). Under basal conditions in striatal neurons *in vivo* or *in vitro*, phosphorylation of DARPP-32 at Thr-75 is high, whereas phosphorylation of Thr-34 is low. Increased cAMP levels, through activation of dopamine D1 receptors *in vivo* or in striatal slices, or through the use of forskolin in striatal slices, leads to increased phosphorylation of Thr-34, but decreased phosphorylation of Thr-75. Through the use of protein phosphatase inhibitors, our results have suggested a mechanism whereby cAMP may activate a PP2A-like protein phosphatase leading to dephosphorylation of Thr-75 of DARPP-32. A number of other studies have also suggested the possibility that cAMP may be able to stimulate dephosphorylation of proteins, in addition to the normally accepted role of cAMP as an activator of protein phosphorylation (11–15).

PP2A is ubiquitously expressed in eukaryotic cells, where it exists as a heterotrimeric enzyme composed of a 36-kDa catalytic C subunit, a 64-kDa scaffolding A subunit, and multiple regulatory B subunits that are thought to influence enzyme activity, substrate specificity and subcellular localization (16–22). Studies have indicated that several B56 [also termed PR61 or PPP2R5, (23–25)] subunit isoforms are phosphorylated in intact cells (23, 26). In addition, *in vitro* studies have found that the B56δ subunit of PP2A is phosphorylated by PKA, resulting in activation of the native heterotrimer (27). Studies have suggested that expression of B56 is high in brain (23, 24). In preliminary studies we found that the 74-kDa B56δ isoform was highly expressed in striatal tissue, raising the possibility that the B56δ subunit might play a role in the regulation of DARPP-32 dephosphorylation by PP2A. Here, we report that PKA activates PP2A via phosphorylation of the B56δ subunit, and that this mechanism is responsible for cAMP-dependent dephosphorylation of Thr-75 of DARPP-32.

Results

PKA Phosphorylates the B56δ Subunit of PP2A *in Vitro* and in Intact Cells. The B56δ subunit of PP2A, expressed in Sf9 cells and purified, was phosphorylated *in vitro* by PKA (Fig. 1*b*). Phosphorylation reached a stoichiometry of ≈4 mol/mol after incubation of B56δ with PKA for 60 min in the presence of okadaic acid (data not shown). Studies have identified Ser-53, Ser-68, and Ser-566 as potential sites of phosphorylation for PKA [(27); Fig. 1*a*]. Mutation of these three sites substantially reduced the phosphorylation of the B56δ subunit (Fig. 1*b*). Additional mutation of another consensus PKA site at Ser-81 (to generate a "quad" mutant) further reduced phosphorylation of B56δ close to baseline levels. We next generated phospho-specific antibodies to each of the four potential phosphorylation sites [see

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Abbreviations: PP2A, protein phosphatase-2A; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; Cdk5, cyclin-dependent kinase 5; AAV, adeno-associated virus; dbcAMP, dibutyryl-adenosine 3-,5-cyclic monophosphate.

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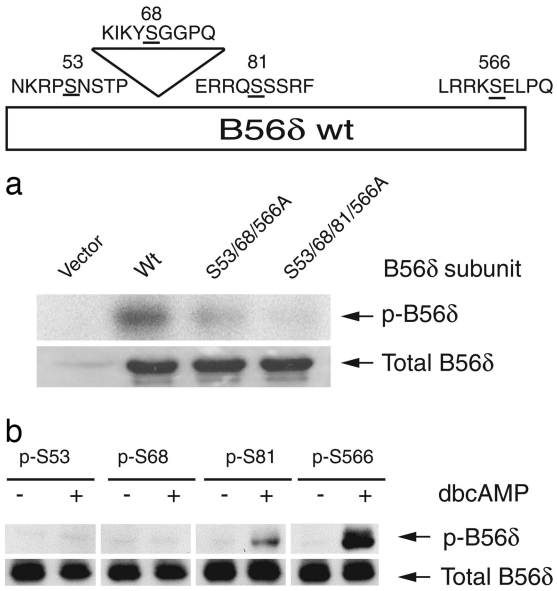

Fig. 1. B56δ is phosphorylated *in vitro* and in intact cells by PKA. The schematic shows the position of sites in rat B56δ phosphorylated *in vitro* by PKA. The peptide sequences illustrated were used to generate phospho-specific antibodies specific for each site (see SI Fig. 8). (*a*) The wild-type (wt) and S55/68/566A and S56/68/81/566A mutant forms of the B56δ subunit were expressed and purified from Sf9 insect cells by using baculovirus. Proteins (10 ng per lane) were incubated with PKA and [^{32}P]ATP for 3 h at 30°C, and reactions were analyzed by SDS/PAGE (4–20% acrylamide), staining with Coomassie blue and autoradiography. (*b*) Flag-B56δ subunit was transfected into HEK293 cells, cells were treated without or with dibutyryl cAMP (dbcAMP, 100 μM) for 30 min, and B56δ subunit was immunoprecipitated with agarose-conjugated anti-FLAG antibody. The phosphorylation of each site (as indicated) was analyzed with its respective phospho-specific antibody. Total B56δ was analyzed by immunoblotting using anti-Flag antibody.

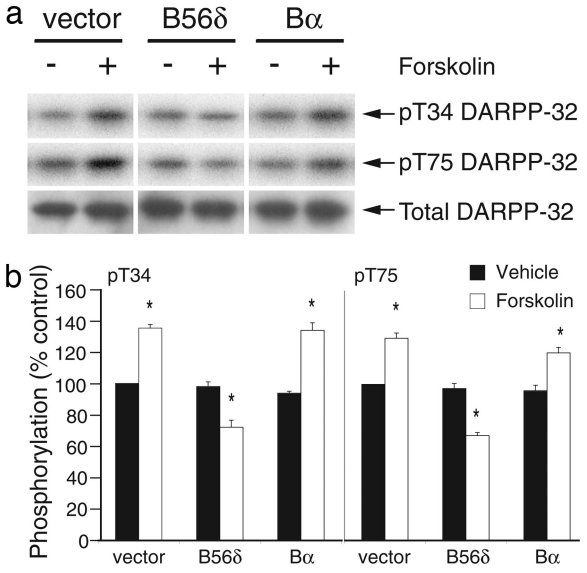

Fig. 2. Exogenously expressed B56δ subunit regulates the dephosphorylation of DARPP-32 in HEK293 cells. (*a*) Cells were transfected with Myc-DARPP-32 without or with B56δ or Bα subunit. Cells were treated with forskolin (10 μM) for 30 min, and the phosphorylation at Thr-34 or Thr-75 of DARPP-32 was analyzed by immunoblotting using phospho-specific antibodies corresponding to each site. Total DARPP-32 expression was analyzed by using anti-Myc antibody. The expression levels of B56δ or Bα were very similar, and similar amounts of heterotrimeric complex were formed by both subunits (data not shown). (*b*) The phosphorylation levels of Thr-34 (*Left*) and Thr-75 (*Right*) were normalized to values obtained for untreated cells expressing only DARPP-32. Data represent means ± SEM ($n = 3$) *, $P < 0.01$ compared with untreated, DARPP-32-only cells, Student's *t* test.

supporting information (SI) Fig. 8]. The B56δ subunit was then transiently expressed in HEK293, after which cells were incubated in the absence or presence of the cAMP analogue dibutyryl cAMP. Dibutyryl cAMP treatment resulted in a marked increase in the phosphorylation of Ser-566 and slightly stimulated Ser-81 phosphorylation (Fig. 1*c*). Phosphorylation of Ser-566 occurred rapidly, reaching a plateau at 5 min after activation of PKA with forskolin (data not shown).

Phosphorylation of the B56δ Subunit Mediates cAMP-Dependent Dephosphorylation of DARPP-32. We next investigated the effects of the B56δ subunit on the regulation of PP2A activity in HEK293 cells. In initial studies, we examined whether exogenous B56δ would form a trimeric complex with the C and A subunits of PP2A. HEK293 cells were transfected with FLAG-tagged wild-type B56δ subunit or with B56δ in which the four phosphorylation sites were mutated to alanine in various combinations. The B56δ subunit was then immunoprecipitated with anti-FLAG antibodies. The wild-type and mutant B56δ proteins each coimmunoprecipitated both the C and A subunits (SI Fig. 9).

To address the role of B56δ subunit in the regulation of DARPP-32 phosphorylation, we transfected DARPP-32 and the B56δ subunit together into HEK293 cells (which do not express any detectable levels of either DARPP-32 or the B56δ subunit, data not shown). Cells were then incubated in the absence or presence of forskolin to activate PKA, and the phosphorylation levels of Thr-34 and Thr-75 of DARPP-32 were analyzed by immunoblotting (Fig. 2 *a* and *b*). As expected, DARPP-32 phosphorylation at Thr-34 was stimulated by forskolin in cells transfected with a vector control. Unexpectedly, Thr-75 phosphorylation was also increased after forskolin treatment of vector control cells (via an unknown mechanism). However, in cells transfected with the B56δ subunit, forskolin treatment reduced phosphorylation of both Thr-34 and Thr-75 of DARPP-32. Confirming a specific role for the B56δ subunit, expression of the Bα subunit of PP2A had no effect on the ability of forskolin to stimulate DARPP-32 phosphorylation. Confirming the role of PP2A in the dephosphorylation of DARPP-32, pretreatment with okadaic acid (1 μM for 60 min) abolished the effect of the B56δ subunit on DARPP-32 dephosphorylation (data not shown). Mutation of the Ser-53, Ser-68, Ser-81, and Ser-566 to alanine (quad mutant) also abolished the effect of the B56δ subunit to mediate forskolin-dependent dephosphorylation of both Thr-34 and Thr-75 of DARPP-32 (Fig. 3).

We next examined the ability of the B56δ subunit to influence PP2A activity *in vitro* in two complementary assays (Fig. 4). In the first assay, wild-type B56δ or the quad mutant were transfected into HEK293 cells. Cells were then incubated without or with forskolin, after which the B56δ subunit was immunoprecipitated and PP2A-dependent dephosphorylation of DARPP-32 (at either Thr-34 or Thr-75) was measured *in vitro*. In the second assay, wild-type or mutant B56δ was transfected into HEK293 cells, after which the B56δ subunit was immunoprecipitated and incubated *in vitro* without or with PKA plus MgATP. PP2A-dependent dephosphorylation of DARPP-32 was then also measured *in vitro*. Either preincubation with forskolin (Fig. 4*a*) or phosphorylation with PKA (Fig. 4*b*) was able to activate PP2A ≈2- to 3-fold toward either phospho-Thr-34 or phospho-Thr-75. However, forskolin pretreatment or phosphorylation by PKA had no effect on PP2A activity associated with the coimmunoprecipitated quad B56δ mutant.

Using the second assay method with phosphoThr34-DARPP-32 as substrate, we also examined the effect of PKA/

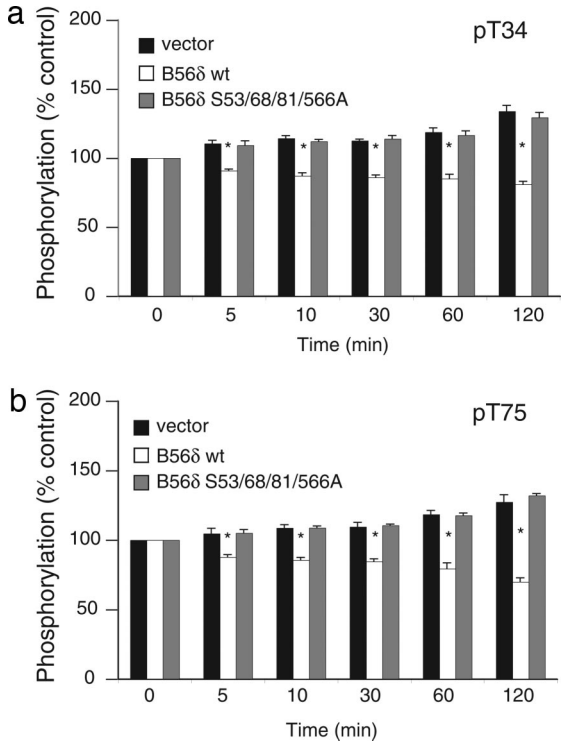

Fig. 3. Phosphorylation of B56δ subunit regulates PP2A-dependent dephosphorylation of DARPP-32. HEK293 cells were cotransfected with Myc-tagged DARPP-32 and either vector, wild-type (wt) B56δ, or mutant B56δ in which Ser-53, 68, 81, and 566 were mutated to Ala (B56S53/68/81/566A). Cells were treated with forskolin (10 μM) for the indicated times, and the phosphorylation and total levels of DARPP-32 were analyzed by immunoblotting (data not shown). The phosphorylation levels of Thr-34 (*a*) and Thr-75 (*b*) were normalized to values obtained at zero time. Data represent means ± SEM ($n = 3$) *, $P < 0.01$ compared with untreated, DARPP-32-only cells, Student's *t* test.

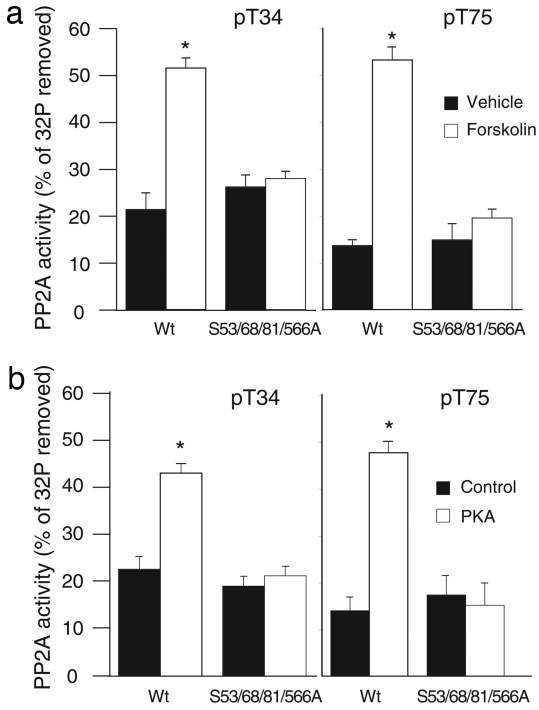

Fig. 4. Phosphorylation of B56δ by PKA activates PP2A. (*a*) HEK293 cells were transfected with either wild-type or mutant Flag-B56δ (as indicated) and cells incubated without (vehicle, DMSO, filled bars) or with forskolin (10 μM, open bars) for 30 min. Cells were lysed and B56δ immunoprecipitated by using agarose-conjugated anti-FLAG antibody. Recombinant DARPP-32 phosphorylated at either Thr-34 (*Left*) or Thr-75 (*Right*) were used as substrates for the immunoprecipitated PP2A. The results are expressed as the percentage of total ^{32}P released from either substrate. Data represent means ± SEM ($n = 3$) *, $P < 0.01$ compared with untreated control, Student's *t* test. (*b*) HEK293 cells were transfected as in *a* in the absence of any cell treatment. The B56δ subunit was immunoprecipitated with anti-Flag antibody, and samples were incubated without (filled bars) or with (open bars) PKA *in vitro*. PP2A activity was measured as in *a*. Data represent means ± SEM ($n = 3$) *, $P < 0.01$ compared with unphosphorylated PP2A, Student's *t* test.

MgATP on the K_m and V_{max} of immunoprecipitated, B56δ-containing, PP2A. Phosphorylation of B56δ increased the V_{max} from 49 to 77.5 μmol/min/mg and reduced the K_m from 46 to 14 μM, resulting in an overall increase in catalytic efficiency of ≈5-fold.

Phosphorylation of Ser-566 of the B56δ Subunit by PKA Is Necessary for Activation of PP2A. To measure the contribution of each phosphorylation site in the B56δ subunit to the regulation of PP2A activity, we expressed mutant proteins in which only one of the four sites was available for phosphorylation. Each mutant (plus wild type and the quad mutant for comparison) was cotransfected with DARPP-32 in HEK293 cells and the phosphorylation of Thr-34 and Thr-75 measured after incubation without or with forskolin. In complementary studies, wild-type and mutant proteins were expressed without DARPP-32, the B56δ subunit was immunoprecipitated and incubated *in vitro* without or with PKA plus MgATP. The results from studies of DARPP-32 phosphorylation in HEK293 cells (see SI Fig. 10), or from *in vitro* PP2A assays (Fig. 5 *a* and *b*), indicated that phosphorylation of Ser-556 by PKA was necessary and sufficient for activation of PP2A toward DARPP-32.

Dominant-Negative Effect of the B56δ Quad Mutant Expressed in Striatal Neurons. We next examined whether phosphorylation of the B56δ subunit is regulated in striatal neurons after activation of dopamine D1 receptors. Mouse striatal brain slices were incubated without or with the D1 receptor agonist SKF38393 (Fig. 6). The B56δ subunit was phosphorylated to a measurable level under basal conditions, and SKF38393 treatment increased phosphorylation of Ser-566 ≈2-fold within 2 min.

This result indicated that the phosphorylation of the B56δ subunit was regulated in striatal neurons by a physiologically relevant stimulus. To further analyze the physiological role of B56δ phosphorylation *in vivo*, we used an adeno-associated virus (AAV) to express either the wild-type or quad mutant forms of B56δ in mouse striatum *in vivo*. Fourteen days after bilateral stereotactic injection, the flag-tagged wild-type B56δ subunit or the quad mutant was stably expressed at levels 1- to 2-fold higher than endogenous B56δ in striatum as measured by immunoblotting (SI Fig. 11). Confirming the results shown in Fig. 6, the D1 receptor agonist SKF81297 increased phosphorylation on Ser-566, but had no effect on the phosphorylation of the other three sites in the B56δ subunit (SI Fig. 12). The effect of expression of the wild-type protein or the quad mutant on the phosphorylation of DARPP-32 was then examined in striatal slices stimulated without or with SKF81297 (Fig. 7 *a* and *b*). In slices previously infected with control virus, or virus encoding either wild-type or mutant B56δ, phosphorylation of DARPP-32 at Thr-34 was increased by SKF81297. In contrast, in slices infected with control virus, or virus encoding wild-type B56δ, SKF81297 treatment resulted in a significant decrease in phosphorylation of Thr-75. However, expression of the B56δ quad mutant attenuated SKF81297-mediated dephosphorylation of Thr-75.

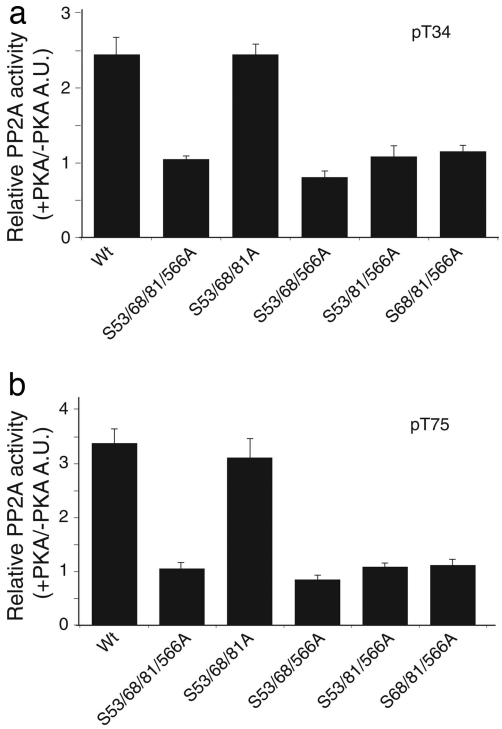

Fig. 5. Phosphorylation of Ser-566 by PKA regulates PP2A activity. HEK293 cells were transfected with wild-type B56δ or mutant B56δ in which individual phosphorylation sites were mutated (as indicated). The B56δ subunit was then immunoprecipitated with anti-Flag antibody, and samples were incubated without or with PKA *in vitro*. PP2A activity was measured by using DARPP-32 phosphorylated at Thr-34 (*a*) or Thr-75 (*b*). Data are expressed as the relative activation of PP2A activity in the absence or presence of phosphorylation by PKA. Data represent means ± SEM ($n = 3$).

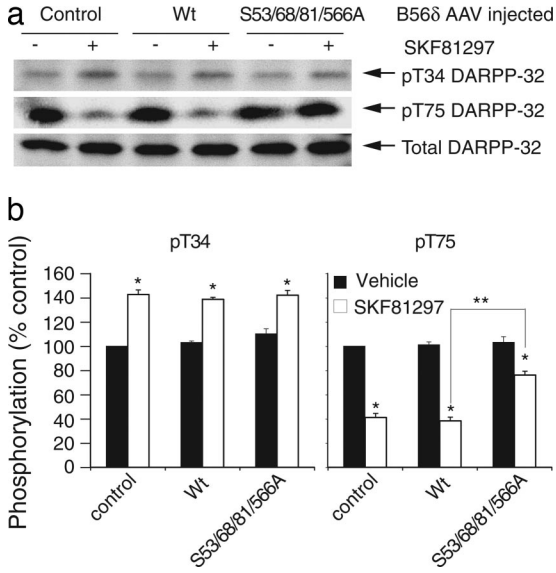

Fig. 7. Expression of a nonphosphorylatable form of B56δ in mouse striatum attenuates the cAMP-dependent dephosphorylation of Thr-75 of DARPP-32. (*a*) By using AAV-mediated infection, wild-type or mutant Flag-B56δ (B56δS53/68/81/566A) was expressed in mouse striatum after stereotaxic injection. The control used empty AAV. After 14 days, striatal slices were prepared and incubated without or with SKF81297 (10 μM for 5 min), and the phosphorylation and total levels of DARPP-32 were analyzed by immunoblotting as described above. (*b*) The phosphorylation levels of Thr-34 (*Left*) and Thr-75 (*Right*) were normalized to values obtained for untreated slices. Data represent means ± SEM ($n = 3$); *, $P < 0.01$ compared with untreated samples from control striatal slices, Student's *t* test. **, $P < 0.01$ comparing B56δ and B56δS53/68/81/566A in the presence of SKF81297.

Discussion

The results obtained in this study suggest a mechanism whereby PP2A is activated by PKA-dependent phosphorylation of the B56δ subunit, present as part of the heterotrimeric PP2A assembly. Phosphorylation of Ser-566 of the B56δ subunit is responsible for activation of PP2A, and this site is found to be regulated in intact cells in response to increased cAMP generation. These studies expand on a previous report that indicated that the B56δ subunit was phosphorylated *in vitro* by PKA (27). Our previous studies have shown by using striatal slices that activation of D1 receptors leads to cAMP-dependent dephosphorylation of Thr-75, the Cdk5 site in DARPP-32 (10). Our present results indicate that PP2A activity is increased up to 5-fold by phosphorylation of the B56δ subunit. Moreover, a mutant B56δ subunit that cannot be phosphorylated by PKA, acts in a dominant-negative manner to block D1-mediated dephosphorylation of Thr-75 after expression in striatal neurons. These results support the conclusion that a D1/cAMP/PKA/PP2A-dependent pathway is responsible for dephosphorylation of Thr-75 in striatal neurons. This pathway would act to attenuate the inhibitory constraint that phospho-Thr-75 has on the phosphorylation of Thr-34 of DARPP-32 by PKA as part of a positive feedback mechanism and would amplify dopaminergic signaling through the D1/PKA/Thr-34-DARPP-32/PP1 signal transduction cascade (see also refs. 2 and 10).

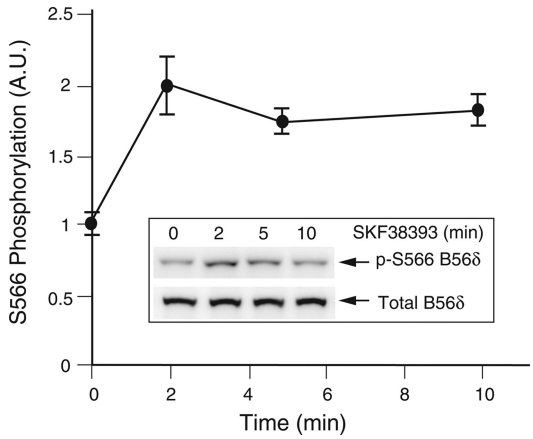

Fig. 6. B56δ is phosphorylated at Ser-566 by PKA in striatal slices in response to activation of D1 dopamine receptors. Mouse striatal slices were incubated with SKF38393 (10 μM) for various times (as indicated), and phosphorylation of Ser-566 in B56δ was measured by immunoblotting (see *Inset* for representative experiment). Ser-566 phosphorylation was normalized to total B56δ levels, and then values were normalized to that obtained at zero time in each experiment. Results shown represent the average from three experiments.

Together with PP1, PP2A accounts for a large amount of the serine/threonine protein phosphatase activity in all eukaryotic cells, and PP2A is known to play a role in virtually all aspects of cellular function (17, 18). PP2A activity is controlled through the interactions of the catalytic C subunit with the scaffolding A subunit and the regulatory B subunits. Four major classes of B subunit exist (the B, B′ (B56), B″, and B‴ isoforms), which are believed to dictate the substrate specificity of each specific PP2A heterotrimer. Up to 10 B56 isoforms are generated by 5 genes, with all B56 isoforms containing a highly conserved central region of ≈400 aa (23, 24). A feature of the different B56 subunits is the variability in their N- and C-terminal regions. The 4 phosphorylation sites that we have identified in B56δ are all

found in these variable regions (Fig. 1; and see ref. 23). Ser-53 and Ser-68 are not conserved in other B56 isoforms, whereas Ser-81 is conserved in B56γ1, -γ2, -γ3, and B56δ, with Ser-566 also being found in B56γ2 and -γ3. Notably, Ser-81 and Ser-566 were the two sites found to be regulated in intact cells. The variable presence of these phosphorylation sites suggests that PP2A, containing different B56 isoforms, will be subject to differential control by cAMP/PKA or possibly other kinases. In this respect, a recent study has found that B56δ plays an important role in PP2A-mediated regulation of Cdc25, thereby controlling cell cycle progression in response to DNA checkpoints (28). Ser-37 of *Xenopus* B56δ (equivalent to Ser-53 in rat B56δ) was found to be phosphorylated by Chk1 in a checkpoint-dependent manner leading to enhancement of PP2A holoenzyme formation.

An interesting question is how does phosphorylation of Ser-566 influence PP2A activity. The recent elucidation of the crystal structure of a C/A/B56γ heterotrimer has provided important insight into holoenzyme assembly, and the role of the B56 subunit in substrate recognition (29). Surprisingly, the central core of the B56 subunit contains a number of HEAT-like repeats, despite having no amino acid sequence similarity to conventional HEAT domains. The HEAT-like elements make multiple contacts with the C subunit, suggesting mechanisms whereby the specificity of substrate interactions with the active site of PP2A can be determined. The truncated B56γ subunit used in that structural study did not contain the C-terminal region encompassing Ser-566. However, it seems likely that the N- and C-terminal regions of B56 isoforms will have additional influence on PP2A activity. For example, N-terminal deletion of B56γ1 can affect substrate specificity (30). Phosphorylation of Ser-566 of B56δ may be able to increase PP2A affinity for DARPP-32 or other substrates through additional protein–protein interactions, or by affecting the structure of the B56 core which in turn influences the interaction of the catalytic domain with substrates.

In the current study, we found that the heterotrimer of PP2A containing the B56δ subunit could effectively dephosphorylate both Thr-34 and Thr-75 of DARPP-32, in HEK293 cells as well as *in vitro*. The ability of the B56δ/PP2A heterotrimer to efficiently dephosphorylate Thr-34 was somewhat unexpected although our previous studies have found that phosphorylation of Thr-34 can be increased by treatment with okadaic acid implying a likely role for PP2A (31). However, in striatal slices mutant B56δ subunit that could not be phosphorylated by PKA, acted in a dominant-negative manner to block only D1-mediated dephosphorylation of Thr-75. We interpret these results as suggesting that in the environment of the striatal medium spiny neuron where DARPP-32 is expressed at very high levels, the balance between PKA-mediated phosphorylation and PKA/PP2A-mediated dephosphorylation favors the kinase reaction after D1 receptor activation. It seems likely that PKA/PP2A-dependent dephosphorylation may contribute a negative feedback role that would contribute to the transient phosphorylation of Thr-34 of DARPP-32 observed in medium spiny neurons in response to D1 receptor activation (10).

Our studies focused on the dephosphorylation of DARPP-32 by B56δ/PP2A but this heterotrimeric form of the phosphatase is likely to have many additional physiological substrates (17, 28). Previous *in vitro* studies by Usui *et al.* found that PKA phosphorylation could activate the B56δ/PP2A heterotrimer by using histone H1 as substrate, but that there was little effect by using Histone H2B or phosphorylase a as substrate (27). Together with our data, these results suggest that the effect of phosphorylation of B56δ by PKA may vary depending on the particular substrate analyzed. Interestingly, our recent studies have suggested that Ser-97, the site in DARPP-32 phosphorylated by CK2, may also be subject to cAMP/PKA-dependent regulation of B56δ/PP2A (A. Stipanovich, E. Valjent, M. Sanchez y Matamales, A.N., J-H.A., K. Brami-Cherrier, H. Enslen, A.C.N., P.G., D. Hervé, and J.-A. Girault, unpublished results).

A few other studies have suggested that PP2A can be activated by forskolin (14, 32), a drug normally associated with activation of adenylyl cyclase and generation of cAMP. However, those studies suggested an indirect role for forskolin that did not depend on PKA, and perhaps did not depend on cAMP. Clearly, additional research is needed to further characterize whether PP2A is regulated by other cAMP-dependent mechanisms. The B56δ subunit exhibits a widespread tissue distribution being found in most tissues examined, with highest levels being found in brain (33, 34). The B56γ2 and -γ3 isoforms are also widely distributed (23). Although the regulation of B56 by PKA-dependent phosphorylation found here may be limited to certain isoforms and restricted to certain substrates, the present results suggest that activation of PP2A may be widespread and represent an important and unexpected addition to known cAMP-mediated signaling pathways.

Methods

Additional detailed procedures and reagents are described in *SI Experimental Procedures*.

Immunoprecipitation of PP2A Complex and Measurement of PP2A Activity. Transfected cells were lysed in buffer containing 50 mM Tris, pH 8.0, 138 mM NaCl, 27 mM KCl, 1% Triton X-100, protease inhibitor mixture, and phosphatase inhibitor mixture (cocktails 1 and 2; Calbiochem, San Diego, CA). Lysates (500 μg of total protein) were incubated with 50 μl (50% slurry) of anti-FLAG conjugated agarose for 4 h at 4°C. Immunocomplexes were washed three times in lysis buffer without protease and phosphatase inhibitors and two times in PP2A reaction buffer (20 mM Hepes, pH 7.4/150 mM NaCl/5 mM $MgCl_2$/0.0125% Triton X-100). In some cases, immunocomplexes were resuspended in 100 μl of PP2A reaction buffer and incubated without or with PKA catalytic subunit (100 ng) and nonradioactive ATP for 30 min at 30°C, and the reactions were terminated by adding a peptide that inhibits PKA (PKI, 100 μM). Reaction mixtures were washed three times with PP2A reaction buffer and resuspended in 50 μl of PP2A reaction buffer. PP2A activity was measured by using purified DARPP-32-His phosphorylated by using [^{32}P]ATP either at Thr-34 by PKA or at Thr-75 by Cdk1 (a gift from Laurent Meijer, Centre National de la Recherche Scientifique, France) (8). Routine reactions contained 3 μM substrate, and incubations were carried out for 5 min at 30°C. Kinetic assays used 0.3–3 μM substrate and were carried out for 1 min to ensure linear reaction conditions. Samples were analyzed by SDS/PAGE (12% acrylamide) and autoradiography. Results were quantified by densitometry by using National Institutes of Health IMAGE 1.52 software (Bethesda, MD).

Stereotaxic Surgery. All mice used in this study were handled in accordance with the requirements of The Rockefeller University Institutional Animal Care and Use Committee. Male mice (8 weeks) were anesthetized by using 2.5% Avertin solution, their heads were shaved, and the mice were mounted into a stereotaxic apparatus (Kopf, Tujunga, CA). The scalp was cut to reveal the skull, and measurements were taken to ensure that the skull was flat. To target the striata, syringe needles (Hamilton, Reno, NV) were targeted to +0.5 (anterior/posterior), ±2.0 (lateral), and −3.5 (dorsal/ventral) relative to the bregma. A total of 0.5 μl of purified virus (1×10^{12} particles per ml) was delivered by using a micropump (Kopf) over a 2-min period, followed by 5 min of rest. Needles were removed and the scalp incision was closed with wound clips. After 2 weeks, mice were killed, and the phosphorylation status of DARPP-32 in striatum was analyzed by experiments by using striatal slices.

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