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Phosphorylation on the PPP2R5D B regulatory subunit modulates the biochemical properties of protein phosphatase 2A

*Un Young Yu & Jung-Hyuck Ahn**
Department of Biochemistry, Ewha Womans University School of Medicine, Seoul 158-710, Korea

To characterize the biochemical properties of the PP2A regulatory B subunit, PPP2R5D, we analyzed its phosphorylation sites, stoichiometry and effect on holoenzyme activity. PPP2R5D was phosphorylated on Ser-53, Ser-68, Ser-81, and Ser-566 by protein kinase A, and mutations at all four of these sites abolished any significant phosphorylation in vitro. In HEK293 cells, however, the Ser-566 was the major phosphorylation site after PKA activation by forskolin, with marginal phosphorylation on Ser-81. Inhibitory tyrosine phosphorylation on Tyr-307 of the PP2A catalytic C subunit was decreased after forskolin treatment. Kinetic analysis showed that overall PP2A activity was increased with phosphorylation by PPP2R5D phosphorylation. The apparent Km was reduced from 11.25 μM to 1.175 μM with PPP2R5D phosphorylation, resulting in an increase in catalytic activity. These data suggest that PKA-mediated activation of PP2A is enabled by PPP2R5D phosphorylation, which modulates the affinity of the PP2A holoenzyme to its physiological substrates. [BMB reports 2010; 43(4): 263-267]

INTRODUCTION

Protein phosphatase 2A (PP2A) is expressed ubiquitously in eukaryotic organism where it functions as a heterotrimeric enzyme composed of a catalytic C subunit (36 kDa, alpha or beta isoform), a structural A subunit (64 KDa, alpha or beta isoform), and multiple regulatory B subunits. The B regulatory subunits have four protein families according to their amino acid sequence homology, and more than 18 different B subunit genes have been identified. The B subunits have been reported to modulate function of the PP2A holoenzyme by regulating substrate binding, enzyme activity, and subcellular localization (1-3).

In regulatory B subunit-specific manner, PP2A participates in several biological signaling processes. The PPP2R5D subunit plays a critical role in several systems; it regulates Cdc2 release from 14-3-3 in mitosis, and dephosphorylates DARPP-32 which controls the reward system to cocaine addiction in the brain. Among the four major phosphorylation sites, Ser-53 (corresponds to Ser-37 in *Xenopus*) is phosphorylated by Chk1 and modulates the mitotic process, plus it acts as a central checkpoint effector (4). Cocaine-induced activation of PKA results in phosphorylation on Ser-566, and this phosphorylation activates PP2A. Activated PP2A, in turn, dephosphorylates DARPP-32 protein and regulates its subcellular localization to control the drug-seeking behavior in cocaine-administered mice (5, 6). Another regulation mechanism includes glutamate-induced activation of the PP2A with PPP2R3A via internal Ca^{2+} increase (7) in brain. Moreover, the C subunit phosphorylation on Tyr-307 has been reported to be associated in Alzheimer neurofibillary pathology (8).

We focus here on the PPP2R5D subunit, due to the overall PP2A activity is dependent on the phosphorylation event of this regulatory subunit and relative enrichment in dopaminergic neuronal cells where PKA regulates the PP2A activity with phosphorylation.

RESULTS

Stoichiometric analysis of PPP2R5D subunit phosphorylation

It has been reported that PPP2R5D subunit is phosphorylated by protein kinase A on four serine residues (6). We checked that these four sites were the major phosphorylation sites using purified PPP2R5D subunit from Sf-9 insect cells. We compared the amount of phosphorylation between wild-type and the mutant (S53/68/81/566A) in which the four serine residues (Ser-53, Ser-68, Ser-81, and Ser-566) were replaced with alanine. Mutations on these four sites eliminated most of the PKA-mediated phosphorylation in PPP2R5D (Fig. 1a) *in vitro*. We also examine the kinetics of phosphorylation using the mutant form of PPP2R5D proteins containing only one available phosphorylation site. In the S53/68/81A mutant, for example, three serine residues (Ser-53, Ser-68, Ser-81) were replaced with ala-

*Corresponding author. Tel: 82-2-2650-5712; Fax: 82-2-2652-7846; E-mail: ahnj@ewha.ac.kr

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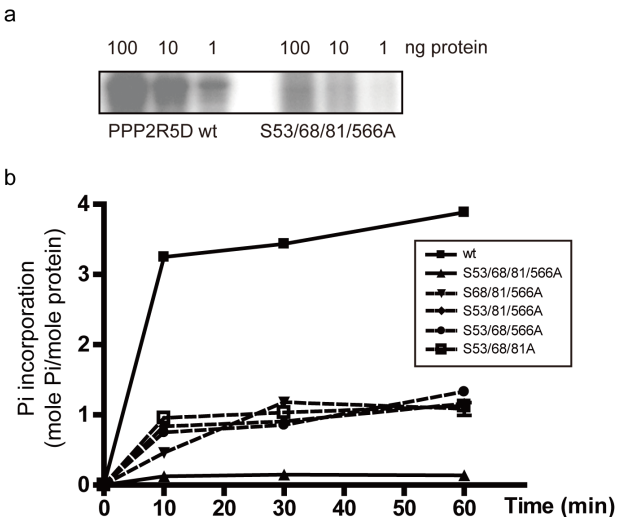

Fig. 1. Stoichiometry of the phosphorylation in the PPP2R5D subunit. (a) Phosphorylation on PPP2R5D subunit by PKA. PPP2R5D wt or S53/68/81/566A mutant proteins were purified from Sf9 insect cells and 1, 10, 100 ng of each protein was incubated with [γ-^{32}P] ATP and 10 ng of PKA catalytic subunit for 10 minutes. Proteins were resolved in 4-20% SDS-PAGE and visualized by autoradiography. (b) The PPP2R5D wt or mutant forms (10 ng) were phosphorylated with [γ-^{32}P] ATP and PKA catalytic subunit for indicated time. The intensity of phosphorylation in wt or each mutant was analyzed by SDS-PAGE, staining with Coomassie blue, and dried. Bands corresponding to PPP2R5D were excised and the cpm was measured using beta-counter.

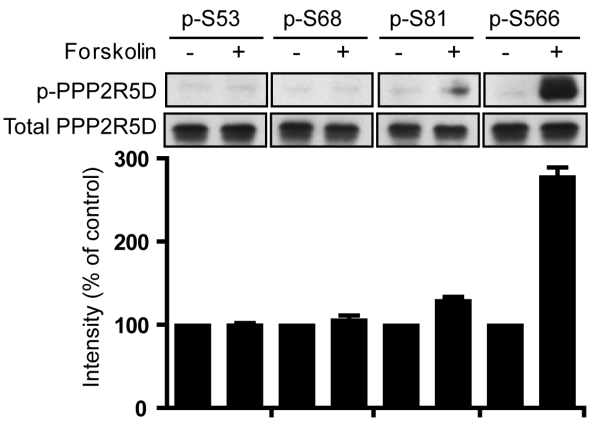

Fig. 2. PKA-activator forskolin induced phosphorylation on PPP2R5D. Flag-tagged PPP2R5D subunit was expressed in HEK293 cells, and then cells were treated with DMSO vehicle or forskolin (10 μM) for 10 minutes. The phosphorylation of each site (Ser-53, 68, 81, and 566) was probed with its respective phospho-specific antibody. Total PPP2R5D was analyzed using anti-Flag antibody. Bar graph showed the quantitation results from the intensity of immunoblotting results normalized to values obtained from untreated cells. Data represent means ± SEM (n = 3).

nine, while the last site (Ser-566) was available for phosphorylation. All four mutant forms of PPP2R5D (S53/68/81A, S53/68/566A, S53/81/566A, and S68/81/566A) showed similar pattern of kinetics with regard to phosphorylation implying that there was no preference by PKA catalytic subunit *in vitro*. We calculated the stoichiometry of phosphorylation using these data (Fig. 1b). The wild-type PPP2R5D was phosphorylated up to 4 molecules of phosphate per molecule of protein, while mutants carrying only one site available for phosphorylation were labeled with approximately one molecule of phosphate. This result indicated that these four sites were the major phosphorylation sites by PKA.

PKA activation induced the phosphorylation on Ser-566 site of the PPP2R5D

There are three different B56 isoforms (includes PPP2R5C γ1, -γ2, -γ3 and PPP2R5D) in which Ser-53 and Ser-68 are not conserved, whereas Ser-81 and Ser-566 are conserved in both PPP2R5C γ2, -γ3, and PPP2R5D (6). Although *in vitro* phosphorylation data showed that PKA had no preferences among these four sites under an experimental condition where only one phosphorylation site was available, we hypothesized that physiological phosphorylation would be differential in certain biological signaling pathways. To identify more physiological phosphorylation sites on the PPP2R5D B subunit by PKA, we transfected HEK293 cells with PPP2R5D expressing plasmid, and the expressed PPP2R5D was incorporated to make heterotrimeric PP2A by interacting with endogenous A/C dimer. We then treated cells with DMSO vehicle or PKA-activator forskolin for 10 minutes. Forskolin activated PKA by increasing the intracellular cAMP level, and the phosphorylation on Ser-566 showed three-fold increase with forskolin treatment (Fig. 2). Phosphorylation on another phosphorylation site, Ser-81, was also marginally increased with forskolin treatment. The increased phosphorylation by forskolin was seen within 5 minutes after treatment implying that this Ser-566 site was physiological substrate of PKA.

Forskolin reduced the inhibitory phosphorylation on Tyr-307 of catalytic subunit

The phosphorylation on Tyr-307 residue of the catalytic C subunit was reported to have an inhibitory effect on the PP2A holoenzyme, while Ser-566 phosphorylation at the PPP2R5D increase the activity (6, 8). We examine the Tyr-307 phosphorylation after PKA activator forskolin treatment to examine the phosphorylation status on this inhibitory site upon PP2A stimulation. HEK293 cells transfected with PPP2R5D wild type (wt) or S53/68/81/566A mutant were treated with forskolin for 10 minutes. Immunoblotting with phospho-Tyr-307 antibody showed decreased phosphorylation on this residue, however, the decrease was seen in both wt and mutant-transfected cells indicating that the phosphorylation on PPP2R5D subunit did not affect C subunit Tyr-307 phosphorylation (Fig. 3).

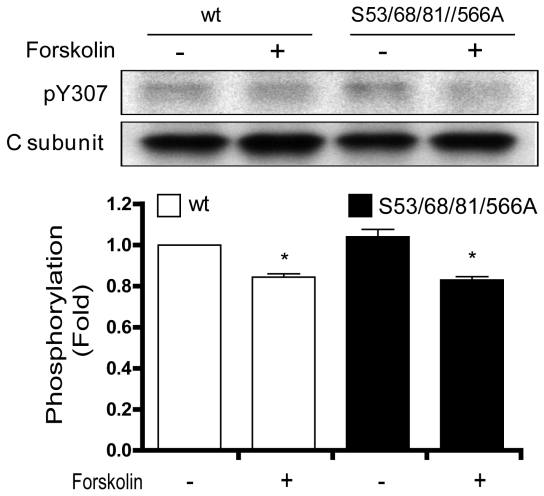

Fig. 3. Forskolin-induced dephosphorylation of Tyr-307 in PP2A catalytic subunit. HEK293 cells were transfected with Flag-tagged PPP2R5D wt or S53/68/81/566A mutant, and then treated with 10 μM of forskolin for 10 minutes, and the phosphorylation on Tyr-307 of catalytic C subunit was analyzed by immunoblotting using phospho-specific antibody. Bar graph represents quantitation results, and normalized to values from wt-transfected vehicle-treated cells. Data represent means ± SEM (n = 3) *P < 0.001 compared with untreated wt cells, one way ANOVA with Newman-Keuls multiple comparison test.

Phosphorylation on PPP2R5D lowers the Km value to increase the activity of the PP2A

A previous study showed that phosphorylation on Ser-566 activated the PP2A towards its physiological substrate dopamine- and cAMP-regulated phosphoprotein 32 kDa (DARPP-32) by three folds. We expressed the Flag-tagged PPP2R5D wild-type or S53/68/81/566A mutant in HEK293 cells, then immunoprecipitated the Flag-PPP2R5D with anti-Flag antibody (Fig. 4a). The exogenously expressed PPP2R5D wild-type and mutant interacted with endogenous A/C dimer, as the dimer was co-immunoprecipitated with PPP2R5D. These data indicated that the phosphorylation of PPP2R5D by PKA did not affect the regulation through C subunit phosphorylation or B subunit binding to A/C subunit. Next, we analyzed the apparent Km and Vmax with immunoprecipitated the PP2A A/C-PPP2R5D complex. HEK293 cells transfected with Flag-tagged PPP2R5D wild-type or S53/68/81/566A mutant were lysed and immunoprecipitated with anti-Flag antibody to pull down the PP2A A/C with Flag-PPP2R5D subunit. The PP2A containing either PPP2R5D wt or S53/68/81/566A subunit was incubated with PKA active catalytic subunit, and as a control reaction, PP2As with wild-type or mutant PPP2R5D were incubated with heat-inactivated PKA. Phosphorylation by PKA decreased the apparent Km value from 11.25 μM to 1.27 μM, and also slightly decreased the Vmax from 41.84 μmol/min/μg to 33.11 μmol/ min/μg, resulting in an overall activation by approximately 7.5-fold (Fig. 4b).

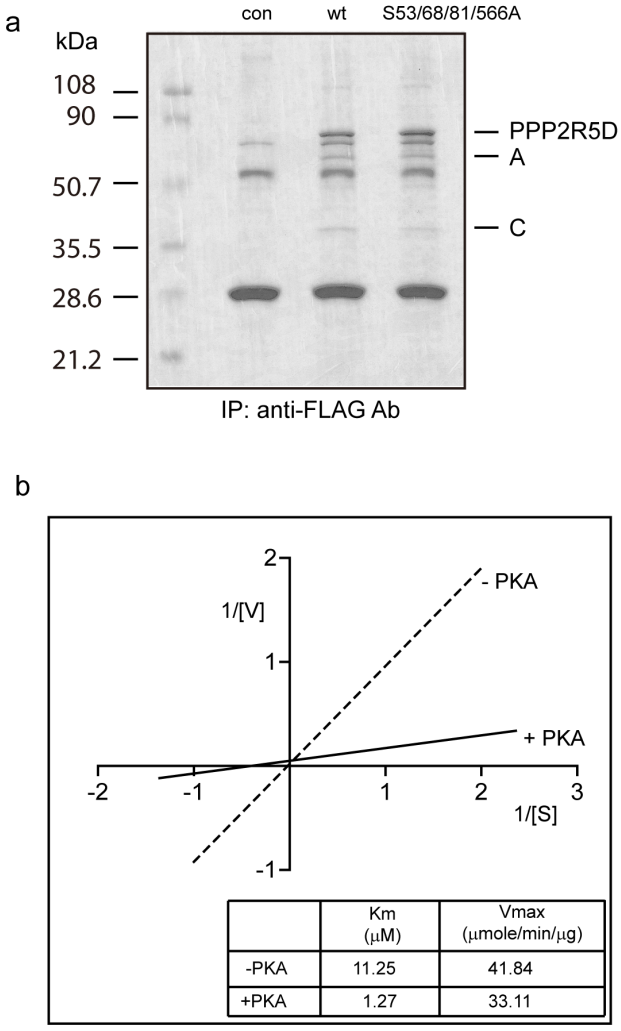

Fig. 4. The effect of PPP2R5D subunit phosphorylation on interaction with A/C and apparent Km values. (a) HEK293 cells were transfected with either Flag-PPP2R5D wt or Flag-S53/68/81/566A. Cells were lysed, and PPP2R5D subunits were immunoprecipitated by using anti-Flag antibodies. Antibody-protein complex were resolved in 4-20% SDS-PAGE and stained with Coomassie blue reagent. (b) Lineweaver-Burk plotting of PP2A with or without phosphorylation. HEK293 cells transfected with wt PPP2R5D subunit were immunoprecipitated by using agarose-conjugated anti-Flag antibody to obtain active PP2A with PPP2R5D subunit. PP2A with PPP2R5D was incubated with ATP and either active PKA (+PKA) or boiled PKA (-PKA) for 10 minutes. PKA activity was blocked by adding PKI inhibitor peptide, and then 0.6, 1.2, or 3 μm of p-Thr-75 DARPP-32 protein was added for a substrate.

DISCUSSION

In this paper, we examine the biochemical effects of phosphorylation in the regulation of the PP2A activity through PPP2R5D regulatory subunit. Forskolin-mediated activation of PKA resulted in an increase of Ser-566 phosphorylation which has

been known as a PP2A-activating process in this subunit. Also, forskolin also reduced inhibitory phosphorylation on Tyr-307 in catalytic C subunit. Taken together, the PP2A activation by PKA includes both a phosphorylation on B subunit and dephosphorylation on C subunit revealing cooperative modulation for controlling the PP2A activity. The effect of phosphorylation on the PPP2R5D subunit was measured by kinetic analysis using partially purified PP2A containing PPP2R5D subunit. PKA-mediated phosphorylation on the PPP2R5D subunit increased overall PP2A activity by lowering the Km value towards its physiological substrate protein DARPP-32.

The four major phosphorylation sites were phosphorylated by PKA *in vitro* without any preference to specific sequence (6, 9), and Rho GTPase-specific kinase (ROCK) also phosphorylated these four sites with a similar pattern as PKA (data not shown) indicating that all four sites were good substrates for several kinases *in vitro*. The physiological role of phosphorylation on each site, however, seems different depending on the particular system in which PP2A is engaged. PKA-activator forskolin strongly enhanced the phosphorylation level on Ser-566, while only slightly induced Ser-81 phosphorylation. The phosphorylation on Ser-566 residue was enough to activate PP2A holoenzyme, and the mutant that had only Ser-566 site available showed similar kinetics to wild-type protein (data not shown). In addition to B subunit regulation, phosphorylation on PP2A catalytic C subunit modulates the activity of PP2A, and the biological importance of this modification has been reported (10, 11). Inhibitory phosphorylation on C subunit Tyr-307 was decreased, whereas PP2A-activating PPP2R5D phosphorylation was increased. The methylation on C subunit determined the interaction of PR55/B subunit family to PP2A A/C dimer, and phospho-Tyr 307 affected binding to distinct B subunit families. The interaction between PPP2R5D with A/C dimer, however, was not affected by phospho-Tyr 307 because pull down experiment for Flag-PPP2R5D using anti-Flag antibody from HEK293 cells treated without or with forskolin yielded same amount of A or C subunit co-immunoprecipitation. Forskolin treatment increased intracellular cAMP which triggers several biochemical signaling pathways, and interestingly, enhanced the PP2A activity via two separated targets (12-14).

Kinetic analysis indicated that the mechanism of PP2A activation with phosphorylated PPP2R5D was due to a decrease in the Km value. The regulatory B subunit is considered to specify the physiological substrates and to modulate the PP2A holoenzyme. Phosphatase assay of the PP2A with PPP2R5D subunit revealed that activity change of PP2A was largely dependent on the specific substrates used. Small phosphorylated oligo-peptides were less effective for measuring activation folds than physiological substrate phospho-DARPP-32, confirming the role of the B subunit in its substrate specificity. Recent data from our laboratory revealed that specific protein kinase C specific inhibitors such as Go6976 or Rottlerin (15) appeared to modulate the phosphorylation status of the PPP2R5D subunit, implying that complicated kinase cascades were involved in several biochemical signaling pathways in which PP2A played an important role.

MATERIALS AND METHODS

Stoichiometric analysis of PPP2R5D phosphorylation

6XHistidine tagged-PPP2R5D subunit wild-type and phosphorylation sites mutants were purified from Sf9 insect cells with serial chromatography using Ni-NTA column (Amersham, NJ) and gel filtration column. Purified PPP2R5D proteins were incubated with PKA catalytic subunit for indicated time. We used DARPP-32 mutant protein in which Thr-75 was replaced by alanine (T75A) as a standard for calculating incorporation of Pi into PPP2R5D. To prepare labeled substrate, 10 μg of purified DARPP-32 T75A mutant protein was phosphorylated with 10 of PKA catalytic subunit and 5 μl of fresh [γ-^{32}P] ATP (10 mCi/ml) in 50 μl reaction at 30°C for 1 hour. Our previous study confirmed that this process was enough to make more than 1 : 0.9 (protein: Pi) radioactive Pi incorporation ratios (16). After phosphorylation reaction of PPP2R5D by PKA, proteins were resolved in 4-20% SDS-PAGE, dried, and PPP2R5D bands were excised then radioactivity (count per minute) was measured with beta-counter (Beckman). The standard was used to calculate the stoichiometry of radioactive Pi incorporation into PPP2R5D proteins according to the molecular weight ratios (72 kDa PPP2R5D: 32 kDa DARPP-32).

Transfection and immunoblotting

HEK293 cells were grown in DMEM containing 10% FBS with penicillin-streptomycin in humidified CO_2 incubator. Expression plasmids for Flag-tagged PPP2R5D wild-type or mutant were transfected into HEK293 cells with Lipofectamine 2000 reagent (Invitrogen). Cells were treated with 10 μM of forskolin for 10 minutes at forty-eight hours post-transfection. Cells were lysed and proteins were resolved in 4-20% gradient SDS-PAGE and transferred to nitrocellulose membrane. Immunoblotting was performed with anti-phospho-PPP2R5D antibodies corresponding to each phosphorylation site.

Kinetic analysis

His-tagged PPP2R5D subunit was purified from Sf9 insect cell using baculovirus protein expression system (Clontech) with several modifications (17). PP2A A/C subunit complex were purified using His-tagged A subunit and C subunit co-infected into Sf9 cell. His-tagged A subunit formed an A/C complex inside the Sf9 cells, and this complex was purified using Ni-NTA Hi-trap column (Amersham). Purified PPP2R5D subunit and A/C complex were mixed in PP2A reaction buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.0125% Triton X-100), and incubated with either active PKA catalytic subunit (+PKA) or heat-inactivated PKA catalytic subunit (-PKA) for 10 minutes. These reactions were stopped by adding PKA-inhibiting peptide (PKI, 100 μM). PP2As either with phospho- or unphospho- PPP2R5D subunits were then mixed with 0.6, 1.2, or

3 μM of 32-P labeled DARPP-32 substrates phosphorylated by CDC2 kinase. After 5 minutes, each reaction was terminated by adding 2X SDS-PAGE sample buffer. Proteins were resolved with 4-20% SDS-PAGE, then the DARPP-32 protein bands were excised and the count per minutes (cpm) was measured with beta-counter (Beckman).

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