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Activation of protein phosphatase 2A by cAMP-dependent protein kinase-catalyzed phosphorylation of the 74-kDa B″ (δ) regulatory subunit in vitro and identification of the phosphorylation sites

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Abstract Human erythrocyte protein phosphatase 2A, which comprises a 34-kDa catalytic C subunit, a 63-kDa regulatory A subunit and a 74-kDa regulatory B″ (δ) subunit, was phosphorylated at serine residues of B″ in vitro by cAMP-dependent protein kinase (A-kinase). In the presence and absence of 0.5 μM okadaic acid (OA), A-kinase gave maximal incorporation of 1.7 and 1.0 mol of phosphate per mol of B″, respectively. The K_m value of A-kinase for CAB″ was 0.17 ± 0.01 μM in the presence of OA. The major in vitro phosphorylation sites of B″ were identified as Ser-60, -75 and -573 in the presence of OA, and Ser-75 and -573 in the absence of OA. Phosphorylation of B″ did not dissociate B″ from CA, and stimulated the molecular activity of CAB″ toward phosphorylated H1 and H2B histones, 3.8- and 1.4-fold, respectively, but not toward phosphorylase *a*.

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Key words: Protein phosphatase 2A; 74-kDa regulatory subunit; Phosphorylation sites; cAMP-dependent protein kinase; Human erythrocyte

1. Introduction

Protein phosphatase 2A (PP2A), one of the four major classes of protein-serine/threonine phosphatases, is widely distributed among eukaryotes, and plays an important role in the regulation of diverse cellular processes such as metabolism, signal transduction, DNA transcription, translation, cell cycle and cell proliferation [1–3]. Previously [4,5], we purified three forms of PP2A from human erythrocyte cytosol, whose subunit structures are CAB″ ($\alpha_1\beta_1\delta_1$), CAB ($\alpha_1\beta_1\gamma_1$) and CA ($\alpha_1\beta_1$). They have a common subunit structure, CA, where C is a 34-kDa catalytic subunit and A is a 63-kDa regulatory subunit. Binding of another regulatory subunit, either 53-kDa B or 74-kDa B″, to CA changed the substrate specificity and metal ion requirement of CA [5]. Recently, based on partial amino acid sequences of B″, cDNA of B″ was cloned from a human cerebral cortex cDNA library [6]. B″ has strong sequence similarity to the B′ regulatory subunit family of PP2A [7–10], especially in the middle 400-amino acid region of the molecules, and these are comprised in the B′ family. The central conserved region of the molecule is supposed to interact with the CA core structure of PP2A, and diverse N- and C-termini are considered to affect phosphatase activity, substrate specificity, or localization of the enzyme. In the predicted primary structure of B″, a number of consensus phosphorylation sites for cAMP-dependent protein kinase (A-kinase) and C-kinases were found [6]. Previously, we observed in vitro phosphorylation of CAB″ at serine residues of B″ by A-kinase with concomitant stimulation of the phosphatase activity toward phosphorylated histones [11]. Rat brain 72-kDa B″ in CAB″ was also phosphorylated by A-kinase in vitro to the same extent as human erythrocyte 74-kDa B″ [12]. McCright et al. [9] reported in vivo phosphorylation of the B′/B″ subunits of PP2A at their serine residues in cultured cells transfected with the epitope-tagged human B′/B″ constructs. A *Saccharomyces cerevisiae* homologue of B′/B″, Rts1, was also phosphorylated in vivo [13].

In this paper, we identified three in vitro phosphorylation sites of B″ in CAB″ by A-kinase. These phosphorylation sites were located in the divergent N- and C-terminal regulatory regions. The B″ phosphorylation changed the substrate specificity of CAB″ without dissociating B″ from CA.

2. Materials and methods

2.1. Materials

Human erythrocyte CAB″ ($\alpha_1\beta_1\delta_1$) and CA ($\alpha_1\beta_1$), catalytic subunits of pig heart A-kinase, ^{32}P-H2B histone, ^{32}P-H1 histone and ^{32}P-phosphorylase *a* were prepared as described previously [5].

2.2. Phosphorylation reaction

Phosphorylation of CAB″ (29.3 units, 35.2 μg) by catalytic subunits (6.4 units, 2 μg) of A-kinase was carried out for 2 h at 30°C in four separate 90-μl reaction mixtures containing 20 mM HEPES-NaOH, pH 7.4, 5 mM $Mg(CH_3COO)_2$, 0.025%(v/v) Triton X-100, 0.5 mM dithiothreitol, 10 μM [γ-^{32}P]ATP (500 cpm/pmol, Amersham) and 0.5 μM okadaic acid (OA) (Wako) in polypropylene tubes. The reaction was stopped by adding 30 μl of SDS-sample buffer, followed by boiling for 3 min. The boiled sample was subjected to 10% SDS-PAGE [5]. The gel was stained with Coomassie blue, dried and autoradiographed. Phosphorylated B″ were excised from the gel, and the ^{32}P-radioactivity was measured by counting Cerenkov radiation.

2.3. Lysyl endopeptidase digestion of ^{32}P-labeled B″

^{32}P-labeled B″ (15 μg, 1.2 mol phosphate per mol of B″) in hydrated gel pieces was homogenized with a disposable plastic homogenizer (Kontes) in 500 μl of 50 mM Tris-HCl, pH 9.0, then digested with shaking for 24 h at 37°C by adding 1:40 (w/w) lysyl endopeptidase

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Abbreviations: PP2A, protein phosphatase 2A; A-kinase, cAMP-dependent protein kinase; P-H2B histone, H2B histone phosphorylated by A-kinase; P-H1 histone, H1 histone phosphorylated by A-kinase; OA, okadaic acid; AMP-PNP, adenosine 5′-(β,γ-imido)-triphosphate; PTH, phenylthiohydantoin; NLS, nuclear localization sequence

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(Wako) four times. During the incubation, the buffer was changed twice. The supernatant taken from the gel suspension was further incubated at 37°C with newly added 1:40 (w/w) lysyl endopeptidase in parallel with the digestion of ^{32}P-labeled B″ in gel suspension. In total, 1:6.7 (w/w) lysyl endopeptidase was used for the digestion. All supernatants were combined and lyophilized.

2.4. HPLC of phosphopeptides

The lyophilized phosphopeptides derived from ^{32}P-labeled B″ were dissolved in 0.8 ml of 5% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (solvent A), and filtered with a Millipore filter (0.45 μm). The filtrate was applied to a C-18 column (25×0.46 cm, TSK gel ODS-120T, Tosoh) attached to an HPLC system, previously equilibrated with solvent A. The column was developed with an increasing gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.4 ml/min. The gradient was 5% acetonitrile for 10 min, 5–17.5% acetonitrile over 10 min, 17.5–27% acetonitrile over 80 min, 27–40% acetonitrile over 30 min. Fractions of 0.4 ml were collected in polypropylene tubes and the ^{32}P radioactivity in fractions was measured by counting Cerenkov radiation.

2.5. Amino acid sequence analysis

The N-terminal amino acid sequences of the phosphopeptides were determined by Edman degradation on a Hewlett-Packard protein sequencer G1000A. About 20–40 pmol of phosphopeptides was subjected to analysis.

2.6. Gel filtration of phosphorylated CAB″

Phosphorylation of CAB″ (5.1 units, 6.1 μg) by catalytic subunits (17.0 units, 5.3 μg) of A-kinase was carried out for 1 h at 30°C in a polypropylene tube containing 1 ml reaction mixture as described in Section 2.2 except that OA was excluded. After phosphorylation, the reaction mixture was immediately applied to a Superdex 200 column (118×1.0 cm) which was equilibrated with 20 mM HEPES-NaOH, pH 7.4, 0.0125% Triton X-100, 0.15 M KCl, 0.5 mM dithiothreitol, 10% (v/v) glycerol and 0.1 mM phenylmethylsulfonyl fluoride. Gel filtration was performed upward with the same buffer at a flow rate of 16 ml/h at 4°C and fractions of 1 ml were collected.

2.7. Phosphatase assay and protein determination

Protein phosphatase activity was assayed in a 50-μl mixture comprising 20 mM HEPES-NaOH, pH 7.4, 0.15 M KCl, 5 mM $Mg(CH_3COO)_2$, 0.0125% Triton X-100, 100 μM ^{32}P-H2B histone and enzyme preparation in a polypropylene tube. After incubation for 10 min at 30°C, a 45-μl aliquot was withdrawn and transferred to a glass tube containing 1 ml of 5 mM silicotungstic acid and 2.5 mM H_2SO_4 to stop the reaction. The measurement of [^{32}P]Pi release was performed as described previously [5]. One unit of the enzyme was defined as the amount of enzyme which catalyzed the release of 1 nmol of [^{32}P]Pi per min.

Protein was determined by the method of Bradford [14] with bovine serum albumin as standard.

3. Results and discussion

When three forms, CAB″, CAB and CA, of human erythrocyte PP2A were incubated separately with [γ-^{32}P]ATP and catalytic subunits of A-kinase, only B″ in CAB″ was phosphorylated [11]. The stoichiometry of phosphorylation of B″ in CAB″ by A-kinase was 1.7 and 0.4 mol phosphate per mol of B″ in the presence and absence of 0.5 μM OA, respectively

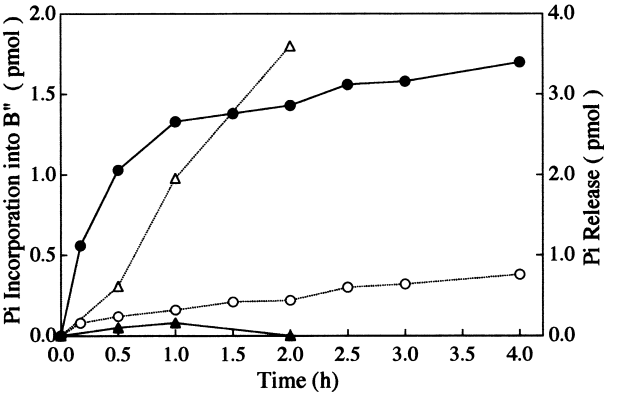

Fig. 1. Time course of ^{32}Pi incorporation into B″ by A-kinase and ^{32}Pi release during the incubation. Phosphorylation of CAB″ (150 milliunits, 180 ng) by catalytic subunits (32 milliunits, 10 ng) of A-kinase was carried out at 30°C in a 30 μl reaction mixture in the presence (●, ▲) or absence (○, △) of 0.5 μM OA as described in Section 2. After 2 h of incubation, the same amount of A-kinase was added to the reaction mixture. At indicated times, ^{32}Pi incorporation into B″ (●, ○) and ^{32}Pi release (▲, △) were measured as described in Section 2. Control values without CAB″ and without A-kinase were subtracted from the complete value for ^{32}Pi release measurement.

(Fig. 1). Increased labeling in the presence of OA was due to the inhibition of autodephosphorylation of CAB″ by OA (Fig. 1). The B″ phosphorylation may be regulated by autodephosphorylation to turn off the input signal.

The K_m value of A-kinase for CAB″ was determined to be 0.17 ± 0.01 μM (mean ± S.E.M., $n = 3$) in the presence of 0.5 μM OA. The V_{max} value of phosphorylation was 105.1 ± 0.9 nmol/min per mg of A-kinase. The K_m value is similar to the estimated intracellular concentration (0.2 μM) of CAB″ [11], suggesting that the enzyme could be one of the physiological substrates for A-kinase.

In order to determine the phosphorylation sites, ^{32}P-labeled B″ (15 μg, 1.2 mol phosphate per mol of B″) was isolated by SDS-PAGE and was extensively digested with lysyl endopeptidase, and resultant phosphopeptides were purified by HPLC on a reverse-phase C-18 column. Three major radioactive peptides, denoted by peptides 1, 2 and 3, were eluted at acetonitrile concentration of 20, 21 and 24.5%, respectively (Fig. 2). The N-terminal amino acid sequences of the phosphopeptides were determined by an amino acid sequencer as listed in Table 1. Comparison of these sequences with deduced amino acid sequence of B″ [6] indicates that peptides 1, 2 and 3 correspond to residues 58–70, 74–79 and 573–580 of B″, respectively (Table 1). Since a phosphoserine residue showed no PTH-amino acid signal by peptide sequencing that we employed, and all the unidentified residues matched up to the positions of the serine residue in the expected sequence, the in

Table 1
Determination of in vitro phosphorylation sites in B′ by A-kinase

Peptide	Sequence determined	Sequence expected	Residue phosphorylated
1	RPxNSTPPPTQLS	R^{58}PSNSTPPPTQLSK^{71}	Ser-60
2	YxGGPQ	Y^{74}SGGPQIVK^{82}	Ser-75
3	xELPQDVY	S^{573}ELPQDVYTIK^{583}	Ser-573

Peptides 1, 2 and 3 refer to peaks 1, 2 and 3 in Fig. 2. The N-terminal sequences determined by an amino acid sequencer, the expected peptide sequences obtained by lysyl endopeptidase digestion of B′ and the phosphorylated residues are indicated. x denotes a residue which yielded no PTH-amino acid signal.

vitro phosphorylation sites on B″ by A-kinase were determined to be Ser-60, -75 and -573. Stoichiometry of phosphorylation at Ser-60, -75 and -573 on B″ was 0.29, 0.28 and 0.52 mol phosphate per mol B″, respectively. These phosphorylation sites are preceded by the consensus sequences for the phosphorylation by A-kinase [15]. Among these sites, Ser-573 has a most potential preceding sequence (Arg-Arg-X) for the phosphorylation by A-kinase [15]. Indeed, about half of the radioactivity was incorporated into Ser-573 on B″ by A-kinase in the presence and absence of OA.

Previously, we observed that in vitro phosphorylation of the B″ subunit in CAB″ by A-kinase slightly stimulated its phosphatase activity [11]. In these experiments, phosphorylation and subsequent dephosphorylation reactions were carried out in the same glass tube. Later, we realized that B″ showed a propensity to bind to glass but not polypropylene surfaces. Therefore, the effect of B″ phosphorylation on CAB″ activity was reinvestigated in the conditions that enzyme reactions were performed in polypropylene tubes and phosphatase activity was measured in the buffer containing 150 mM KCl after isolating the phosphorylated enzyme from other constituents by gel filtration.

CAB″ was first incubated with [γ-^{32}P]ATP and catalytic subunits of A-kinase in the absence of OA for 1 h at 30°C, then the reaction mixture was immediately subjected to gel filtration on a Superdex 200 column equilibrated with a buffer containing 150 mM KCl at 4°C (Fig. 3A). By the gel filtration, CAB″ was eluted at fraction 52, and was well separated from CA, A-kinase and ATP. As a control, phosphorylation was carried out with either 200 μM H-8 (Seikagaku Kogyo), a potent A-kinase inhibitor (Fig. 3B), or AMP-PNP (Sigma) instead of [γ-^{32}P]ATP (Fig. 3C). ^{32}P incorporation into B″ was 1.0 and 0.1 mol per mol of B″ in the absence and presence of H-8, respectively. P-H2B histone phosphatase activity was eluted at fraction 52 (Fig. 3A–C). The phosphatase activity in the complete system (Fig. 3A) was about 3 times higher than those in control systems (Fig. 3B,C). The peak of ^{32}P radioactivity incorporated into the enzyme was also eluted at fraction 52 and coincided with that of phosphatase activity (Fig. 3A,B). The ^{32}P radioactivity in the complete system (Fig. 3A) was about 10 times higher than that in the control system (Fig. 3B). When eluates were analyzed by SDS-PAGE, followed by silver staining and autoradiography, three protein bands corresponding to C, A and B″ were detected stoichiometrically in active fractions and ^{32}P radioactivity was found solely in B″ (data not shown). Almost no autodephosphorylation of ^{32}P-labeled B″ took place during a 5-h gel-filtration at 4°C even in the absence of OA (Fig. 3A,B). These results

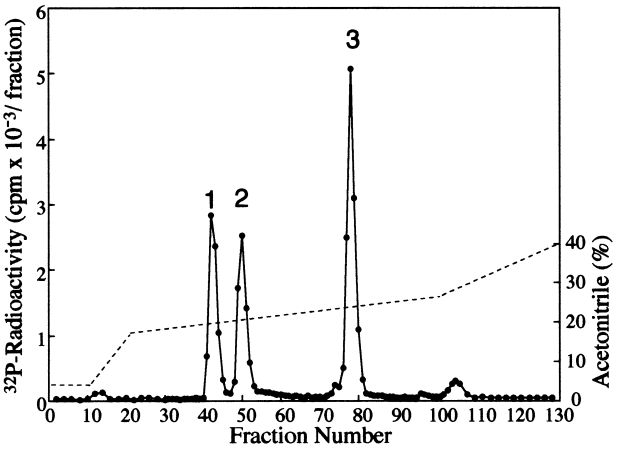

Fig. 2. HPLC of ^{32}P-labeled peptides generated by lysyl endopeptidase digestion of B″ phosphorylated by A-kinase on a C-18 column. Phosphorylation of B″ in CAB″ by A-kinase, isolation of ^{32}P-labeled B″ by SDS-PAGE, digestion of ^{32}P-labeled B″ by lysyl endopeptidase and HPLC of the resultant phosphopeptides were performed as described in Section 2. The overall recovery of ^{32}P radioactivity was 70% of the counts applied to the column. ^{32}P radioactivity (●) and acetonitrile concentration (dotted line) are indicated.

indicate that phosphorylation of B″ by A-kinase stimulates P-H2B histone phosphatase activity of CAB″ without dissociation of B″ from CA (Fig. 3).

To know the effect of B″ phosphorylation on phosphatase activity of CAB″, the kinetic constants of each peak (fractions 50–55 in Fig. 3) were determined using P-H2B histone, P-H1 histone and phosphorylase *a* as substrate (Table 2). Phosphorylation of B″ increased 30–40% the molecular activity of CAB″ toward P-H2B histone with a significant decrease in the K_m value. The molecular activity toward P-H1 histone was increased 3–4-fold by B″ phosphorylation without a change in the K_m value. No significant effect of B″ phosphorylation was detected on the dephosphorylation of phosphorylase *a*. A specificity constant (molecular activity/K_m) value for the dephosphorylation of P-H2B histone and P-H1 histone was increased 6- and 3-fold by B″ phosphorylation, respectively, implying that the phosphorylation has a more profound effect on the dephosphorylation of P-H2B histone than P-H1 histone. These results indicate that the B″ phosphorylation changes the substrate specificity of CAB″. The amount of Pi incorporated into Ser-60, -75 and -573 in the absence of OA, was determined to be 0.03, 0.20 and 0.47 mol per mol B″, respectively, indicating that the phosphorylation of Ser-75 and/or

Table 2
Effect of B′ phosphorylation on K_m value and molecular activity of CAB′

CAB′ incubated with	K_m (μM) and molecular activity (mol Pi released/min/mol enzyme) of CAB′ for					
	P-H2B histone		P-H1 histone		Phosphorylase *a*	
	K_m	Molecular activity	K_m	Molecular activity	K_m	Molecular activity
ATP, A-kinase	34.2 ± 1.3	372 ± 3	153 ± 22	55.6 ± 7.7	24.4 ± 6.7	3.19 ± 0.59
ATP, A-kinase, H-8	140.3 ± 11.2[a]	271 ± 29[b]	131 ± 10	14.6 ± 1.3[a]	20.1 ± 3.7	2.21 ± 0.56
AMP-PNP, A-kinase	109.5 ± 22.5[a]	292 ± 14[c]	141 ± 18	21.8 ± 1.8[a]	18.7 ± 3.8	3.15 ± 0.16

Protein phosphatase activities toward P-H2B histone, P-H1 histone and phosphorylase *a* were measured as described in Section 2 with each peak fraction (fractions 50–55) in Fig. 3. The K_m and V_{max} values were estimated by fitting the data to the Michaelis-Menten equation. The kinetic constants are expressed as means ± S.E.M. Levels of significance by unpaired *t*-test: [a] $P < 0.001$; [b] $P < 0.005$; [c] $P < 0.01$ versus values of phosphorylated forms.

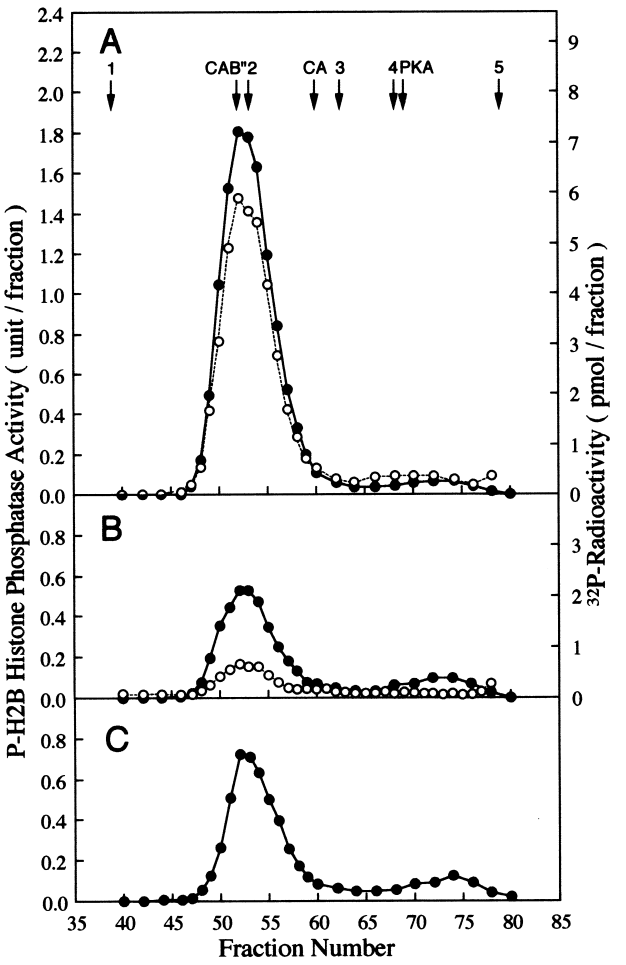

Fig. 3. Gel filtration on Superdex 200 of phosphorylated CAB″. CAB″ (5.1 units, 6.1 μg) was phosphorylated by catalytic subunits (17.0 units, 5.3 μg) of A-kinase in the absence (A) or presence of either 200 μM H-8 (B) or AMP-PNP instead of [γ-^{32}P]ATP (C). Phosphorylation reactions, gel filtration on Superdex 200 and assay for P-H2B histone phosphatase activity (●) were performed as described in Section 2. Since some of the fractions contained ^{32}P radioactivity, a control value without ^{32}P-H2B histone was subtracted from the complete value for phosphatase assay. ^{32}P radioactivity (○) of each eluate fraction was measured by counting Cerenkov radiation of an aliquot of the fraction. Arrows indicate the elution positions of blue dextran (1), γ-globulin (2), serum albumin (3), ovalbumin (4), cytochrome *c* (5), CAB″, CA and A-kinase (PKA). ATP was eluted at fraction 88.

Ser-573 was mainly involved in the activation of CAB″. Comparison of molecular activities of CAB″ and CA toward various substrates revealed that B″ suppressed all the phosphatase activities of CA so far tested [5]; therefore, the B″ phosphorylation by A-kinase appears to counteract the inhibitory effect of B″. One of the physiological significances of the activation of CAB″ by A-kinase-catalyzed B″ phosphorylation could be a shut off mechanism of the A-kinase-mediated signals in the cell.

In the C-terminus of B″, a bipartite nuclear localization sequence (NLS, residues 548–564) was found [6]. Nuclear localization of PP2A [16–18], overexpressed epitope-tagged B56δ (B″) [9] and a 68-kDa protein immunoreactive with specific antisera against B″ [12] has been reported. Since a major phosphorylation site, Ser-573, is located 9 residues C-terminal to the NLS in B″, the reversible phosphorylation of this site may regulate the targeting of PP2A to nucleus. Indeed, phosphorylation in the vicinity of NLSs has been shown to play a role in regulating nuclear protein import through modulation of NLS function [19].

A recent study on the association of B′ in PP2A with cyclin G in vitro and in vivo indicates that PP2A function is regulated by the direct binding of B′ to other regulatory proteins [20]. Since the SH3 domain ligand sequence (residues 523–530) was found in the C-terminus of the B″ subunit [6], B″ phosphorylation at Ser-573 may have some effects on the interaction between B″ and SH3 domain-containing proteins.

In this study, the three in vitro phosphorylation sites of B″ in CAB″ by A-kinase were determined. The B″ phosphorylation changed the activity of CAB″ and its spectrum of substrate specificity. This phosphorylation may also regulate the targeting of CAB″ to a certain compartment in the cell by interaction with a specific protein. In addition to the regulation of PP2A by phosphorylation of the catalytic C subunit [21–24], it is plausible to suggest that the reversible phosphorylation of the regulatory B″ subunit of PP2A serves as another control mechanism of PP2A function in the cell.

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