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Studies on Functions of the 63-kDa A- and 74-kDa B'_δ-Regulatory Subunits in Human Erythrocyte Protein Phosphatase 2A: Dissociation and Reassociation of the Subunits[1]

Rintaro Inoue,[2] Hirofumi Usui, Osamu Tanabe, Yasumasa Nishito, Masahiro Shimizu, and Masao Takeda[3]

Department of Biochemistry, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551

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A heterodimeric form, CA, of protein-serine/threonine phosphatase (PP) 2A purified from human erythrocytes was dissociated into a 34-kDa catalytic subunit C and 63-kDa inactive subunit A by Sephacryl S-200 gel filtration in the presence of 6 M urea. Reassociation of the C- and A-subunits in the absence of urea suppressed the PP activity of the C subunit toward phosphorylase *a*, P-H2B histone, and P-H1 histone in the presence or absence of 20 mM $MnCl_2$ or 50 mM $Mg(CH_3COO)_2$, but stimulated the PP activity toward P-H1 histone in the presence of 200 mM NaCl and the Mn^{2+}-dependent protein-tyrosine phosphatase (PTP) activity toward P-Tyr-Glu copolymers. The 74-kDa inactive B'_δ subunit was isolated from a heterotrimeric form, CAB'_δ, of PP2A partially purified from human erythrocytes, by heparin-Sepharose column chromatography. The B'_δ subunit reassociated with CA and suppressed the PP- and PTP-activities of CA. The B'_δ subunit did not associate with the isolated C subunit directly, and had no effect on the activities of the C subunit, indicating that the A subunit is essential for the association of the B'_δ subunit with CA and the resulting suppression of the PP- and PTP-activities.

Key words: human erythrocytes, protein-serine/threonine phosphatase 2A, reconstruction of subunits, 74-kDa regulatory subunit B'_δ, 63-kDa regulatory subunit A.

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Protein-serine/threonine phosphatase (PP) 2A, one of the four major PPs, distributes ubiquitously in eukaryotic cells (*1*) and plays a central role in the regulation of many cellular events, including metabolism, the cell cycle, cell proliferation, development, replication, transcription, translation, and viral transformation (*1, 2*). Holoenzymes of PP2A have either a heterodimeric or heterotrimeric subunit structure. The heterodimeric structure is composed of a 32-41-kDa catalytic C subunit complexed to a 60-69-kDa regulatory A subunit. The dimeric structure of CA is common to all PP2A holoenzymes. The heterotrimeric structure includes an additional subunit, either 51-58-kDa B (*1*), 54-74-kDa B′/B″ (*1, 3*), or 59-130-kDa PR59/PR72 (*4, 5*).

Previously (*6*), we purified pig heart PP2A (M_r= 171,000), which is composed of a 34-kDa catalytic C (α) subunit, a 69-kDa inactive A (β) subunit, and a 56-kDa inactive B (γ) subunit. The enzyme could be dissociated to its constituent subunits in 6 M urea (*6*). The catalytic C subunit reassociated with the A subunit in the absence of urea with a concomitant change in substrate specificity and the Mg^{2+} requirement for P-H2B histone phosphatase activity (*6*). The B subunit was able to bind to CA, but not to the isolated C subunit, causing suppression of the PP activity toward phosphorylase *a* and glycogen synthase *b*, without significant effect on the PP activity toward phosphorylated histones. Subsequently, we purified three forms of PP2A from human erythrocyte cytosol with subunit structures CA ($\alpha_1\beta_1$), CAB ($\alpha_1\beta_1\gamma_1$), and CAB″ ($\alpha_1\beta_1\delta_1$), where C is a 34-kDa catalytic subunit and A, B, and B″ are 63-, 53-, and 74-kDa regulatory subunits, respectively (*3*). Recent molecular cloning of B″ (δ) (*7*) revealed that this subunit exhibits strong sequence similarity in the central 400-amino-acid region to 54-kDa B′ (*8-10*) and is a member of the B′ family. Therefore, the nomenclature B'_δ, which represents the δ isoform of the B′ family, will be used hereafter instead of the previous nomenclature B″ or δ.

In this paper, the C-, A-, and B'_δ-subunits in human erythrocyte PP2A were isolated from the holoenzymes by either gel filtration in the presence of 6 M urea or heparin-Sepharose column chromatography. The C subunit was

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[2] Department of Internal Medicine (II), Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551

[3] To whom correspondence should be addressed. Tel: +81-82-257-5136, Fax: +81-82-257-5136, E-mail: mtakeda@mcai.med.hiroshima-u.ac.jp

Abbreviations: P-H1 histone, H1 histone phosphorylated by PKA; P-H2B histone, H2B histone phosphorylated by PKA; PKA, cAMP-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; PP, protein-serine/threonine phosphatase; P-spectrin; spectrin phosphorylated by casein kinase II; PTP, protein-tyrosine phosphatase; P-Tyr-Glu copolymers, Tyr-Glu copolymers phosphorylated by c-Yes; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone.

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confirmed to be an active subunit and the A- and B'_δ-subunits were confirmed to be inactive subunits. The A subunit associated with the C subunit and suppressed PP activity with a concomitant increase in the Mn^{2+}-dependent protein-tyrosine phosphatase (PTP) activity. The B'_δ subunit did not associate directly with the isolated C subunit, but could associate with CA through the A subunit. The B'_δ subunit suppressed both PP- and PTP-activities of CA, but had no effect on these activities in the isolated C subunit.

EXPERIMENTAL PROCEDURES

Materials—Calf thymus H1- and H2B-histones, rabbit skeletal muscle phosphorylase *b* and phosphorylase kinase, pig heart PKA and its catalytic subunits, and human erythrocyte spectrin and casein kinase II were prepared as described previously (*3, 11*). c-Yes was prepared from a rat liver plasma membrane fraction (*12*). [γ-^{32}P]ATP was obtained from Amersham. BSA,Tyr-Glu (1:4) copolymers (M_r=49,100), horse heart cytochrome *c*, bovine liver catalase, yeast alcohol dehydrogenase, PMSF, TPCK, human γ-globulin, ovalbumin, and human carbonic anhydrase were obtained from Sigma. Horse spleen ferritin was purchased from Boehringer. Leupeptin, antipain, and pepstatin were from the Peptide Institute. Sepharose 4B, AH-Sepharose 4B, Sephacryl S-200, Superdex 200, and heparin-Sepharose 6B were purchased from Pharmacia. Toyopearl (HW-65, superfine) and DEAE-Toyopearl were obtained from Tosoh. CNBr-activated Toyopearl and H1 histone-Toyopearl were prepared as described previously (*11*). Other chemicals were obtained from various commercial sources.

Preparation of Substrates—^{32}P-H1 histone (17 nmol of bound ^{32}P/mg H1 histone), ^{32}P-H2B histone (63 nmol of bound ^{32}P/mg H2B histone), ^{32}P-phosphorylase *a* (8 nmol of bound ^{32}P/mg phosphorylase *a*), and ^{32}P-spectrin (1 nmol of bound ^{32}P/mg spectrin) were prepared as described previously (*3, 11*). ^{32}P-Tyr-Glu copolymers (123 nmol of bound ^{32}P/mg Tyr-Glu copolymers) were prepared as described previously (*13*). All substrate concentrations represent the concentrations of bound ^{32}P in the substrates.

Phosphatase Assay—Unless otherwise stated, PP activity was measured in a 50-μl mixture containing 50 mM Tris-HCl, pH 7.4, 0.5 mM DTT, 0.05% (v/v) Triton X-100, 200 mM NaCl, 100 μM ^{32}P-H2B histone, and an enzyme preparation (standard assay conditions). PTP activity was measured in a 50-μl mixture containing 50 mM Hepes-NaOH, pH 7.4, 0.5 mM DTT, 0.05% Triton X-100, 50 mM NaCl, 10 mM $MnCl_2$, 0.5 μM ^{32}P-Tyr-Glu copolymers, and an enzyme preparation. The reaction was carried out at 30°C for 5-10 min and terminated by the addition of 1 ml of either 5 mM silicotungstic acid and 2.5 mM sulfuric acid (PP activity) or the same reagent containing 10% (w/v) trichloroacetic acid (PTP activity). The [^{32}P]P_i released was measured as described previously (*3*). One unit of enzyme was defined as the amount of enzyme that catalyzed the release of 1 nmol of [^{32}P]P_i per min.

Preparation of PP2As from Human Erythrocyte Cytosol—A heterodimeric form, CA, of PP2A was purified as described before (*11*). A heterotrimeric form, CAB'_δ, of PP2A was partially purified by sequential column chromatographies on DEAE-Sephadex, DEAE-Toyopearl, AH-Sepharose 4B, and H1 histone-Toyopearl as described previously (*3*). The CAB'_δ preparation had a specific activity of 19 units/mg protein and was purified 8-fold from the peak fraction of DEAE-Toyopearl column chromatography.

Assay for the A- and B'_δ-Subunits—The A subunit was assayed by the stimulative effect on P-H1 histone phosphatase activity of the C subunit in the presence of 200 mM NaCl as previously described (*6*). One unit of A subunit was defined as the amount of subunit that stimulated the release of 1 nmol of [^{32}P]P_i per min. The B'_δ subunit was assayed by the inhibitory effect on P-H2B histone phosphatase activity of the heterodimeric form, CA, of PP2A. Samples were pre-incubated on ice for 10 min with 20 milliunits (with P-H2B histone) of CA in 8 μl of Buffer A [10 mM imidazole-HCl, pH 6.5, 10% (v/v) glycerol, 0.005% Triton X-100, 0.5 mM DTT, and protease inhibitors (0.1 mM PMSF, 1 mM benzamidine, 0.5 μg/ml pepstatin, 0.5 μg/ml antipain, 1 μg/ml leupeptin, and 10 μg/ml TPCK)] containing 250 mM NaCl; then the P-H2B histone phosphatase activity of the reaction mixture was measured under the standard assay conditions. Control incubations, with no sample or with no CA were run simultaneously. The sum of these two control values was subtracted from the final value for the sample with CA. One unit of B'_δ subunit was defined as the amount of subunit that inhibited the release of 1 nmol of [^{32}P]P_i per min. Under these conditions, the inhibition of P-H2B histone phosphatase activity was proportional to the amount of B'_δ subunit up to 10 milliunits, indicating that the B'_δ subunit in the milliunit range of 3-10 can be measured by the procedure (Fig. 1).

Preparation of the C- and A-Subunits—A heterodimeric form, CA, of PP2A (818 units with P-H2B histone, 29.8 units with P-Tyr-Glu copolymers, 0.91 mg, 1.44 ml) purified from human erythrocyte cytosol was mixed gradually with 4.32 ml of 8 M urea within 3 min to a final concentration of 6 M, kept on ice for 10 min, and then applied to a Sephacryl S-200 column (54×2.2 cm) equilibrated with Buffer B (50 mM Tris-HCl, pH 7.4, 10% glycerol, 0.005% Triton X-100, 1 mM DTT, and protease inhibitors) containing 6 M urea. Elution was carried out with the same buffer

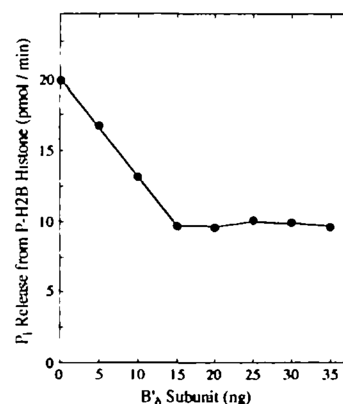

Fig. 1. **Effect of the B'_δ subunit on P_i release from P-H2B histone catalyzed by CA.** The indicated amount of the B'_δ subunit was pre-incubated in Buffer B on ice for 10 min with 20 milliunits of CA; and P_i release from P-H2B histone was measured under the standard assay conditions for PP activity. Details are described under "*Assay for the A- and B'_δ-Subunits*" in "EXPERIMENTAL PROCEDURES."

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at a flow rate of 6 ml/h, and the eluate was passed through a continuous flow countercurrent dialyzing apparatus to remove the urea by dialysis against Buffer B at a flow rate of 70 ml/h. Fractions of 2.3 ml each were collected in glass tubes. The fractions containing the C- and A-subunits, as shown by the solid bars in Fig. 2, were separately pooled, concentrated by an Amicon ultrafiltration cell equipped with a YM-10 filter membrane to a final concentration of 55.6 and 113 μg/ml, respectively, and were stored at −80°C.

Preparation of the B'_δ Subunit and CA—The partially purified CAB'_δ (1,255 units, 66 mg protein, 95 ml) was dialyzed against one liter of Buffer A for 5 h with a change of buffer every hour. The dialyzed enzyme solution (102 ml) was applied to a heparin-Sepharose 6B column (7.5 × 1.6 cm) equilibrated with Buffer A. The column was washed with 140 ml of Buffer A, then the B'_δ subunit and CA were eluted with a 1,340-ml linear 0-0.9 M NaCl gradient in Buffer A. The flow rate was 100 ml/h and 12.5-ml fractions were collected in polypropylene tubes. CA was eluted at 0.04 M NaCl and the B'_δ subunit was eluted at 0.4 M NaCl (Fig. 5). In order to concentrate the pooled B'_δ fractions (192 units, 410 μg, 32 ml), they were diluted with the same volume of Buffer A and applied to a heparin-Sepharose 6B column (2.6 × 0.7 cm) equilibrated with Buffer A containing 0.2 M NaCl. The B'_δ subunit was eluted at a flow rate of 10 ml/h with Buffer A containing 0.5 M NaCl, and 1 ml fractions were collected in polypropylene tubes. The B'_δ subunit was concentrated to 123 μg/ml and stored at −80°C.

Gel Electrophoresis and Immunoblotting—SDS-PAGE and subsequent detection of proteins by either Coomassie Blue staining, silver staining, or Western blotting were performed as described previously (*14*).

Other Determinations—The molecular weight and the frictional ratio (f/f_0) of the dissociated and reconstructed PP2As and subunits were determined by the method of Siegel and Monty (*15*) from the $s_{20,w}$ value and the Stokes radius, with the partial specific volume of 0.725 cm^3/g. The $s_{20,w}$ value was determined by sucrose density gradient centrifugation as described before (*16*) with catalase (11.3 S), alcohol dehydrogenase (7.4 S), and cytochrome *c* (1.9 S) as standards. The Stokes radius was estimated by gel filtration on Sephacryl S-300 or Superdex 200 with ferritin (7.9 nm), human γ-globulin (5.55 nm), BSA (3.5 nm), ovalbumin (2.73 nm), and cytochrome *c* (1.7 nm) as standards under the conditions described in Figs. 3 and 6. Protein was determined by the method of Lowry *et al.* (*17*) or Bradford (*18*) with BSA as a standard.

RESULTS

Isolation of the C- and A-Subunits of PP2A—To dissociate the C- and A-subunits of the heterodimeric form, CA, of human erythrocyte PP2A, purified CA was subjected to gel filtration on Sephacryl S-200 in the presence of 6 M urea (Fig. 2). The C subunit was detected by its PP activity toward P-H2B histone and PTP activity toward P-Tyr-Glu copolymers. The A subunit was assayed for its ability to stimulate the P-H1 histone phosphatase activity of the C subunit in the presence of 200 mM NaCl. Gel filtration

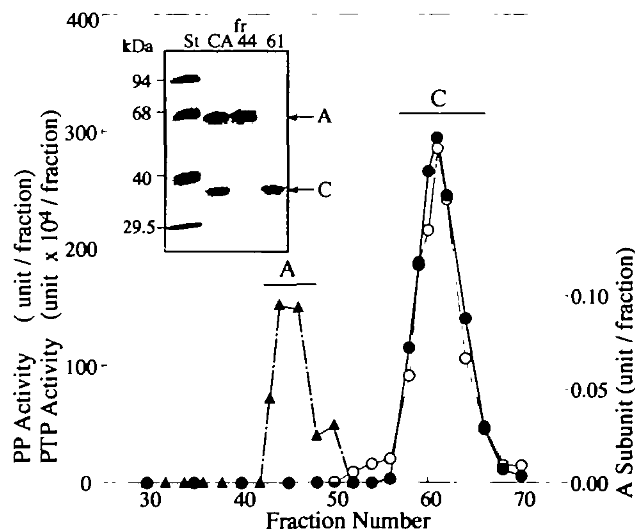

Fig. 2. **Dissociation of the C- and A-subunits.** A heterodimeric form, CA, of PP2A purified from human erythrocyte cytosol was mixed with urea to a final concentration of 6 M. The mixture was kept on ice for 10 min, and subjected to gel filtration on a Sephacryl S-200 column (54 × 2.2 cm) eluted with buffer containing 6 M urea. The urea was immediately removed by passing the eluate through a countercurrent dialyzing apparatus equipped to the exit of the column. Fractions of 2.3 ml each were collected. Details are described under "*Preparation of the C- and A-Subunits*" in "EXPERIMENTAL PROCEDURES." PP activity toward P-H2B histone (●), PTP activity toward P-Tyr-Glu copolymers (○), and the A subunit (▲) were assayed as described under "EXPERIMENTAL PROCEDURES." The inset shows the Coomassie Blue-stained bands on SDS-PAGE of the marker proteins (St), CA, and fractions 44 and 61.

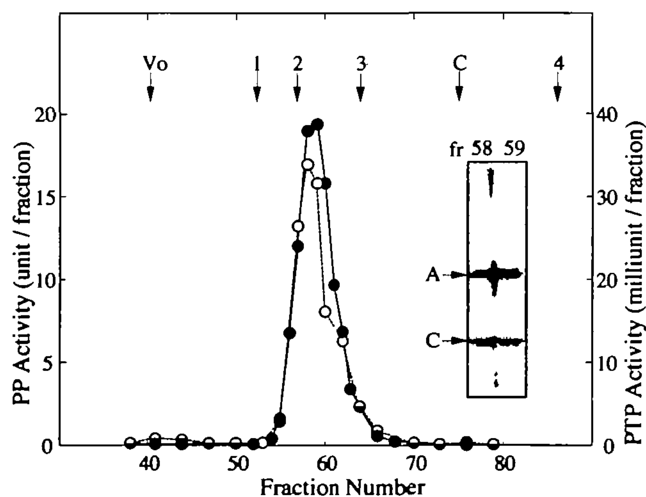

Fig. 3. **Reassociation of the C- and A-subunits.** The C subunit (358 units with P-H2B histone, 46 milliunits with P-Tyr-Glu copolymers, 61 μg, 1.79 nmol, 1.1 ml) and the A subunit (113 μg, 1.79 nmol, 1 ml) were pre-incubated in Buffer B on ice for 30 min and subjected to gel filtration on a Sephacryl S-300 column (50 × 2.2 cm) equilibrated with Buffer B. Elution was performed upward with the same buffer at a flow rate of 12 ml/h. Fractions of 2 ml each were collected. PP activity toward P-H2B histone (●) and PTP activity toward P-Tyr-Glu copolymers (○) were assayed as described under "*Phosphatase Assay*" in "EXPERIMENTAL PROCEDURES." The arrows indicate the elution positions of blue dextran (Vo), ferritin (1), human γ-globulin (2), BSA (3), the C subunit (C), and cytochrome *c* (4). The inset shows the Coomassie Blue-stained bands on SDS-PAGE of the indicated fractions.

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completely separated the C subunit from the A subunit as shown by the Coomassie Blue-stained protein band seen by SDS-PAGE from the peak fraction of each subunit (Fig. 2). The recoveries of PP- and PTP-activities were 203 and 0.58%, respectively.

Reassociation of the C- and A-Subunits—The isolated C- and A-subunits were mixed in Buffer B in the absence of urea at a molar ratio of 1:1, pre-incubated on ice for 30 min (under these conditions, the C- and A-subunits formed CA within 10 min), and subjected to gel filtration on Sephacryl S-300 equilibrated with Buffer B (Fig. 3). A single active peak with an estimated Stokes radius of 4.7 nm was eluted (Fig. 3). The Stokes radius coincided well with that (4.7± 0.2 nm) of CA (*11*). SDS-PAGE of the peak fraction produced 34-kDa C- and 63-kDa A-bands (Fig. 3). Assuming that the staining properties of the C- and A-subunits with Coomassie Blue are equal, the molar ratio of the A- and C-subunits was calculated to be 1.0:1.1 from the relative percent values of the densitometry scan and the molecular weights. These results identified the active peak to be reassociated CA. The recoveries of PP- and PTP-activities were 27.6 and 367%, respectively, suggesting that the binding of the A subunit to the C subunit suppresses the PP activity and stimulates the PTP activity.

Effect of the A Subunit on the PP- and PTP-Activities of the C Subunit—A constant amount of the catalytic C subunit was pre-incubated with increasing amounts of the inactive A subunit on ice for 10 min to form CA. Then, an aliquot of the pre-incubation mixture was assayed for PP activity toward P-H2B histone and Mn^{2+}-dependent PTP activity toward P-Tyr-Glu copolymers (Fig. 4). Increasing concentrations of the A subunit suppressed the PP activity of the C subunit linearly up to a concentration equimolar to the C subunit concentration (Fig. 4). Saturating amounts of A subunit suppressed the PP activity of the C subunit toward P-H2B histone to 9-25% (Fig. 4 and Table I). In contrast, the PTP activity of the C subunit was stimulated linearly by increasing concentrations of the A subunit, and the activity reached a plateau at an A/C molar ratio of about 1 (Fig. 4). The PTP activity of the C subunit was stimulated about 2-fold by the addition of an equimolar or a higher concentration of A subunit (Fig. 4 and Table I). An equimolar concentration of the A subunit also suppressed the PP activities of the C subunit toward phosphorylase *a* and P-H1 histone to 43 and 46%, respectively (Table I). These inhibitory effects of the A subunit were moderated when 10 mM $MnCl_2$ or 20 mM Mg $(CH_3COO)_2$ was present in the assay mixture (Table I). As previously observed for the reassociation of the pig heart C- and A-subunits (*6*), the P-H1 histone phosphatase activity of the human C subunit is also stimulated by the human A subunit when 200 mM NaCl is present in the assay mixture (Table I). Therefore, the effects of the A subunit on the phosphatase activities of the C subunit varied with the substrates and the assay conditions. Under the conditions employed in Table I, the A subunit instantaneously associated with the C subunit, and the effects of the added A subunit on the PP activities of the C subunit toward various substrates were similar to the effects expected from the comparison of the C subunit- and CA-molecular activities towards these substrates (*3*). Although the PTP activity of the C subunit was stimulated 2-4-fold by the addition of the A subunit, the PTP activity of reconstructed CA was obviously lower than that of purified CA. The reason of the low recovery of PTP activity in reconstructed CA is unknown.

Dissociation of CA and the B′$_\delta$ Subunit—During the purification of CAB′$_\delta$ from human erythrocyte cytosol, we unexpectedly found that heparin-Sepharose column chromatography efficiently dissociates CA from the B′$_\delta$ subunit (*3*), and subsequently established a method to obtain the

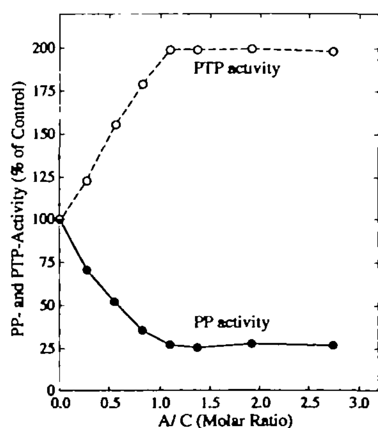

Fig. 4. **Effect of the A subunit on the PP- and PTP-activities of the C subunit.** The C subunit (0.6 unit with P-H2B histone, 0.084 milliunit with P-Tyr-Glu copolymers, 111 ng, 3.27 pmol) was pre-incubated with the A subunit (56.5-565 ng, 0.897-8.97 pmol) on ice for 10 min in a 20-μl mixture containing 20 mM Hepes-NaOH, pH 7.4, 0.005% Triton X-100, 10% glycerol, and 0.5 mM DTT. A 4-μl aliquot of the pre-incubation mixture was assayed for PP activity toward P-H2B histone (●), and for PTP activity toward P-Tyr-Glu copolymers (○) as described under "*Phosphatase Assay*" in "EXPERIMENTAL PROCEDURES."

TABLE I. **Effect of the A subunit on the PP- and PTP-activities of the C subunit.** The C subunit (0.38 unit, 111 ng, 3.27 pmol) and/or the A subunit (206 ng, 3.26 pmol) were pre-incubated on ice for 10 min in a 20-μl mixture containing 20 mM Hepes-NaOH, pH 7.4, 0.5 mM DTT, 0.005% Triton X-100, and 10% glycerol. The PP activity toward phosphorylase *a* (1.6 μM), P-H1 histone (100 μM), and P-H2B histone (100 μM), and the PTP activity toward P-Tyr-Glu copolymers (2 μM) were measured in a 50-μl mixture containing 50 mM Hepes-NaOH, pH 7.4, 0.5 mM DTT, 0.05% Triton X-100, the indicated concentration of each substrate, and 4 μl (in the case of phosphorylase *a* and P-H2B histone) or 20 μl (in the case of P-H1 histone and P-Tyr-Glu copolymers) of the pre-incubation mixture, in the presence or absence of 10 mM $MnCl_2$, 20 mM $Mg(CH_3COO)_2$, or 200 mM NaCl.

Substrate	Additive in assay	P_i release (pmol/min) by			Change in activity (%)
		C	A	C+A	
Phosphorylase *a*	None	6.92	0	2.96	43
	10 mM $MnCl_2$	7.74	0	4.08	53
	20 mM $Mg(CH_3COO)_2$	9.36	0	5.68	61
P-H1 histone	None	20.2	0	9.38	46
	10 mM $MnCl_2$	32.6	0	19.2	59
	20 mM $Mg(CH_3COO)_2$	20.4	0	13.6	67
	200 mM NaCl	19.4	0	25.4	131
P-H2B histone	None	98.6	0	9.20	9
	10 mM $MnCl_2$	150	0	10.4	7
	20 mM $Mg(CH_3COO)_2$	161	0	18.6	12
P-Tyr-Glu copolymers	None	0	0	0	
	10 mM $MnCl_2$	0.145	0	0.338	233

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B'_δ subunit as well as CA from purified CAB'_δ (*14*). To know the effect of the B'_δ subunit on the phosphatase activities of CA, the B'_δ subunit and CA were prepared from CAB'_δ partially purified from human erythrocyte cytosol by column chromatography on heparin-Sepharose 6B with a linear 0-0.9 M NaCl gradient in Buffer A (Fig. 5). A major PP activity peak was eluted at 0.04 M NaCl. The PP activity toward P-H2B histone was stimulated more than 2-fold in the presence of 50 mM Mg^{2+}. It has been shown that the PP activity of CA is stimulated 2-5-fold by 50 mM Mg^{2+} while the activity of CAB'_δ is not stimulated (*3*). Therefore, the major activity peak was suggested to be CA. Gel filtration on Superdex 200 determined the Stokes radius of the major activity peak to be 4.5 nm, which coincides with that of CA (Fig. 6B). Furthermore, SDS-PAGE of the activity peak eluted from Superdex 200 produced Coomassie Blue-stained bands of the C- and A-subunits in a 1:1 molar ratio (data not shown). The B'_δ subunit was eluted at 0.4 M NaCl (Fig. 5) and assayed by the inhibitory effect on the P-H2B histone phosphatase activity of CA. No P-H2B histone phosphatase activity was detected in the B'_δ fractions (Fig. 5). Western blotting with a specific antiserum against the B'_δ subunit (*14*) detected a 74-kDa band only in the B'_δ fractions (fractions 72-76) and not in the active CA fractions (fractions 23-26) eluted with 0.04 M NaCl (Fig. 5). The B'_δ fractions were pooled, applied to a second, smaller heparin-Sepharose column, and concentrated by stepwise elution (see "EXPERIMENTAL PROCEDURES"). SDS-PAGE of the B'_δ preparation produced a major 74-kDa Coomassie Blue-stained band which was estimated to account for 70% of the total stained bands (data not shown).

Reassociation of CA and the B'_δ Subunit—The B'_δ subunit (45.6 units, 73 μg) and CA (88 units, 170 μg), which were dissociated by column chromatography on heparin-Sepharose 6B (Fig. 5), were mixed on ice for 10 min in Buffer A containing 0.4 M NaCl, then subjected to gel filtration on a Superdex 200 column equilibrated with Buffer B containing 0.3 M NaCl. The P-H2B histone phosphatase activity was eluted as a major peak with an estimated Stokes radius of 5.9 nm (Fig. 6A). The $s_{20,w}$ value of the active peak was estimated to be 6.9 S by sucrose density gradient centrifugation. The molecular weight of the reconstructed enzyme was estimated from the Stokes radius and the $s_{20,w}$ value to be 175,000, which coincides with the estimated molecular weight (180,000) of CAB'_δ (*3*). The total P-H2B histone phosphatase activity (32.6 units) recovered from the column was 40% of the total activity of CA mixed with the B'_δ subunit. The low recovery may be attributable to the suppression of CA activity by its binding to the B'_δ subunit. When the concentrated CA fractions from the heparin-Sepharose 6B column were subjected to gel filtration on the same Superdex 200 column, a major activity peak with an estimated Stokes radius of 4.5 nm and a minor activity peak with an estimated Stokes radius of 2.2 nm were eluted (Fig. 6B). These Stokes radii coincided well with those of CA and the C subunit, respectively. The recovery of the total P-H2B histone phosphatase activity (117 units) was 124% of the applied CA activity. The high recovery was probably due to the partial dissociation of the C subunit from CA during the procedures. When the B'_δ preparation alone was subjected to gel filtration on the same Superdex 200 column, the B'_δ subunit was eluted at about the same fraction as the reassociated enzyme (Fig. 6C). The recovery of the B'_δ subunit was 27%. The low recovery may be due to the propensity for the B'_δ subunit to bind to glass surfaces. The Stokes radius and the $s_{20,w}$ value were 5.9 nm and 3.6 S, respectively. The frictional ratio (f/f_0) was estimated to be 1.99, suggesting an unsymmetrical structure.

When P-H2B histone phosphatase activity was measured in the presence of 20 mM $MnCl_2$, the PP activity of the major reassociated enzyme was stimulated (Fig. 6A), while that of the major dissociated enzyme was suppressed (Fig. 6B). To confirm that the reassociated enzyme was CAB'_δ and the dissociated enzyme was CA, Mn^{2+} curves of the P-H2B histone phosphatase activity of these enzymes were compared to those of CAB'_δ and CA (Fig. 7). The effect of

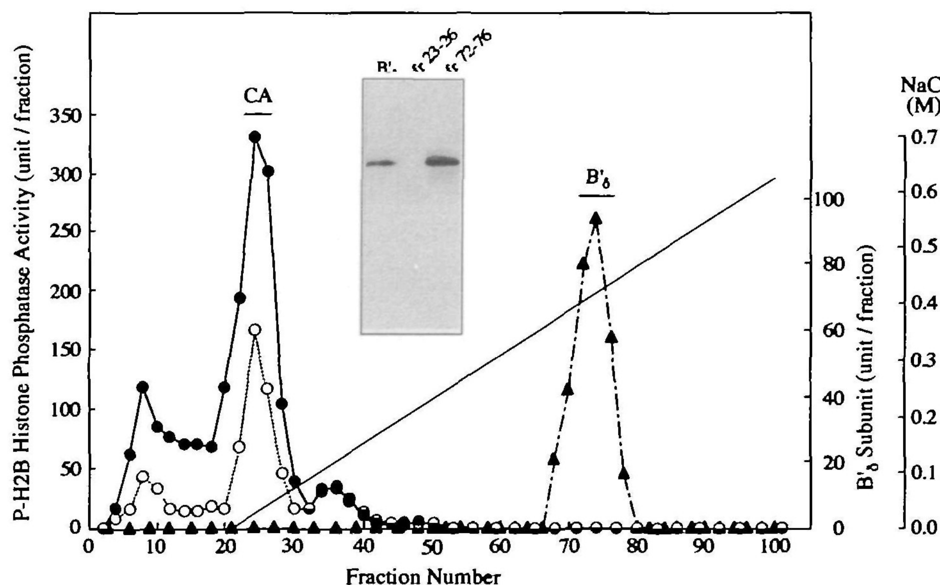

Fig. 5. **Dissociation of the B'_δ subunit and CA.** Partially purified CAB'_δ was applied to a heparin-Sepharose 6B column (7.5×1.6 cm) equilibrated with Buffer A. The B'_δ subunit and CA were eluted with a 1,340-ml linear 0-0.9 M NaCl gradient in Buffer A at a flow rate of 100 ml/h. Fractions of 12.5 ml each were collected in polypropylene tubes. Details are described under "*Preparation of the B'_δ Subunit and CA*" in "EXPERIMENTAL PROCEDURES." PP activity toward P-H2B histone in 2 μl of each fraction was measured in 50 μl of the standard assay mixture except that 0.2 M NaCl was replaced by 50 mM $Mg(CH_3COO)_2$ (●) or H_2O (○). The B'_δ subunit (▲) was assayed as described under "*Assay for the A- and B'_δ-Subunits*" in "EXPERIMENTAL PROCEDURES." The inset shows protein bands detected by Western blotting with an antiserum against the B'_δ subunit after SDS-PAGE of the purified CAB'_δ (B'_δ), pooled fractions 23-26, and pooled fractions 72-76.

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10-40 mM Mn^{2+} on the PP activity was inhibitory with CA and stimulatory with CAB'$_\delta$ (Fig. 7). The Mn^{2+} curves of the dissociated enzyme and the reassociated enzyme were very similar to those of CA and CAB'$_\delta$, respectively (Fig. 7), confirming that the dissociated enzyme is CA, and the reassociated enzyme is CAB'$_\delta$.

Under the same conditions, the B'$_\delta$ subunit (4.5 units, 7.2 μg) and the C subunit (12 units, 1.1 μg) were mixed and subjected to gel filtration on the same Superdex 200 column. The P-H2B histone phosphatase activity was eluted as a single peak with an estimated Stokes radius of 2.2 nm with a recovery of 80% (data not shown). SDS-PAGE of each eluted fraction and subsequent protein silver staining revealed that the 34-kDa C subunit was only detected in the active peak fractions and completely separated from the 74-kDa B'$_\delta$ subunit (data not shown). These results indicate that the B'$_\delta$ subunit can not associate with the C subunit directly but associates through the A subunit.

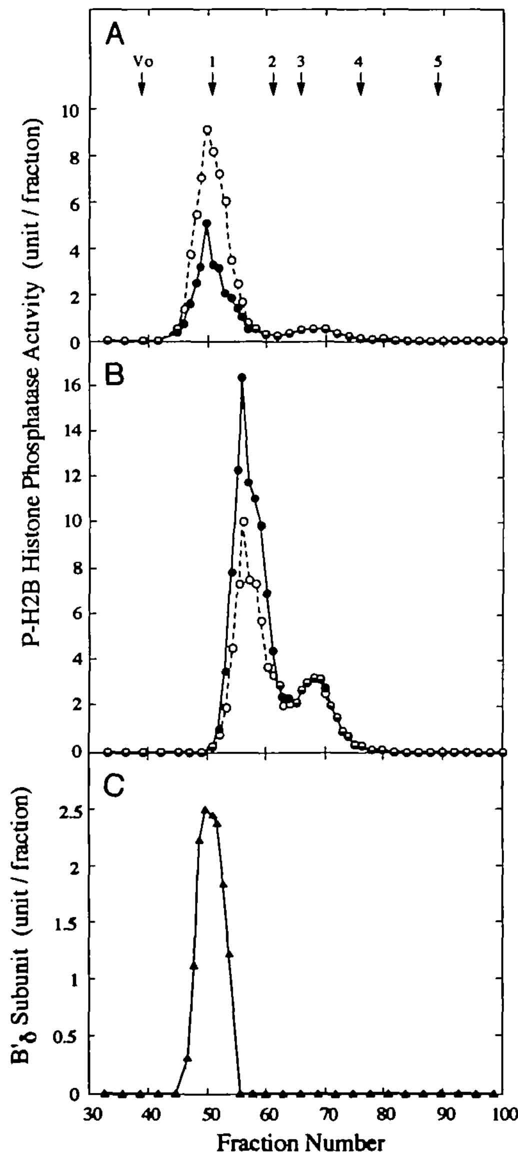

Fig. 6. **Superdex 200 elution profiles for: (A) a mixture of the B'$_\delta$ subunit and CA; (B) CA alone; (C) the B'$_\delta$ subunit alone.** The amount of CA in A and B was 170 μg of protein, 88 units with P-H2B histone, the B'$_\delta$ subunit in A and C was 73 and 84 μg of protein, 45.6 and 52.7 units, respectively. All samples were left standing in 1 ml of Buffer A containing 0.4 M NaCl on ice for 10 min and then separately applied to a Superdex 200 column (118×1 cm) equilibrated with Buffer B containing 0.3 M NaCl. Elution was carried out upward with the same buffer and fractions of 1 ml each were collected at a flow rate of 15 ml/h. PP activity toward P-H2B histone in the absence (●) or presence (○) of 20 mM $MnCl_2$ was measured under the standard assay conditions. The B'$_\delta$ subunit (▲) was assayed as described under "EXPERIMENTAL PROCEDURES." The arrows indicate the elution positions of blue dextran (Vo), human γ-globulin (1), BSA (2), ovalbumin (3), cytochrome *c* (4), and ATP (5).

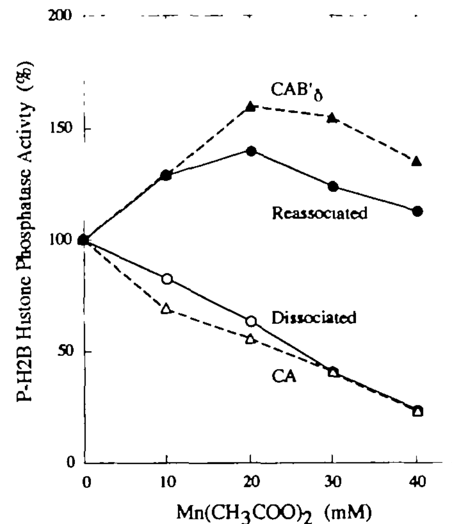

Fig. 7. **Comparison of Mn^{2+} curves of the dissociated- and reassociated-enzymes with those of CA and CAB'$_\delta$.** The P-H2B histone phosphatase activity of fraction 50 (47 milliunits, 7 μl) in Fig. 6A (●), fraction 56 (43 milliunits, 3 μl) in Fig. 6B (○), CAB'$_\delta$ (111 milliunits, 268 ng) (▲), and CA (102 milliunits, 20 ng) (△) was measured under the standard assay conditions except that $Mn(CH_3COO)_2$ at the indicated concentration was included.

TABLE II. **Effect of the B'$_\delta$ subunit on the PP- and PTP-activities of CA.** For the effect on PP activity, CA (17 milliunits, 33 ng) and/or the B'$_\delta$ subunit (12.5 milliunits, 25 ng, 0.23 pmol) were pre-incubated in 5.4 μl of Buffer A containing 0.27 M NaCl in a polypropylene tube on ice for 10 min, and then the PP activities toward P-spectrin (0.5 μM), phosphorylase *a* (20 μM), P-H1 histone (200 μM), and P-H2B histone (100 μM) were measured in a 50-μl mixture under the standard assay conditions except that 0.2 M NaCl was replaced by 0.1 M NaCl and 3 mM $Mg(CH_3COO)_2$. For phosphorylase phosphatase assay, 5 mM theophylline was added to the same reaction mixture. For the effect on PTP activity, CA (170 milliunits) and/or the B'$_\delta$ subunit (50 milliunits) were pre-incubated in 14.7 μl of Buffer A containing 0.3 M NaCl on ice for 10 min and the PTP activity was measured in a 100-μl mixture containing 50 mM Hepes-NaOH, pH 7.4, 0.05 M NaCl, 0.05% Triton X-100, 0.5 mM DTT, 10 mM $MnCl_2$, and P-Tyr-Glu copolymers (0.5 μM). Values are averages of triplicate experiments.

Substrate	P_i release (pmol/min) by			Inhibition by
	CA	B'$_\delta$	CA+B'$_\delta$	B'$_\delta$ (%)
P-spectrin	0.82	0	0.34	59
Phosphorylase *a*	2.99	0	1.57	48
P-H1 histone	1.31	0	1.05	20
P-H2B histone	13.8	0	7.79	44
P-Tyr-Glu copolymers	0.011	0	0.004	64

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TABLE III. **Effect of the A- and B′δ-subunits on the P-H2B histone phosphatase activity of the C subunit.** The C subunit (38 milliunits, 11.1 ng, 0.32 pmol) and/or the A subunit (26.6 ng, 0.41 pmol) and/or the B′δ subunit (25 milliunits, 50 ng, 0.46 pmol) were pre-incubated in 2 μl of Buffer A containing 0.3 M NaCl in a polypropylene tube on ice for 10 min, and the P-H2B histone phosphatase activity was measured in a 50-μl reaction mixture under the standard assay conditions. Values are averages±SD of values from three separate analyses.

Subunit	P_i release (pmol/min) from P-H2B histone
C	38.0±0.5
A	0
B′δ	0
C+B′δ	36.6±0.6
C+A	23.6±1.9
C+A+B′δ	12.4±1.0

Effect of the B′δ Subunit on the PP- and PTP-Activities of CA and the C Subunit—CA was pre-incubated with an equimolar concentration of the B′δ subunit in Buffer A containing 0.27 M NaCl on ice for 10 min to form CAB′δ, then assayed for PP activities toward P-spectrin, phosphorylase *a*, P-H1 histone, and P-H2B histone in the presence of 0.1 M NaCl and 3 mM Mg $(CH_3COO)_2$ (Table II). The B′δ subunit suppressed all the PP activities of CA, although the degree of inhibition varied with the substrate from 20 to 59% (Table II). The same pre-incubation of the C subunit with the B′δ subunit had no effect on the PP activities of the C subunit toward these substrates (data not shown, Table III), consistent with the finding that mixing of the C- and B′δ-subunits under the same conditions does not result in the formation of a CB′δ complex. The complex formation of CAB′δ also suppressed the PTP activity toward P-Tyr-Glu copolymers of CA to 36% (Table II). The substrate specificity of the reconstructed CAB′δ was similar to that of CAB′δ purified from human erythrocyte (data not shown).

Role of the A Subunit in the Inhibitory Effect of the B′δ Subunit on the PP Activity of CA—The P-H2B histone phosphatase activity of the isolated C subunit was not significantly suppressed by pre-incubation with excess B′δ subunit (Table III). The PP activity was suppressed to 62% when the C subunit was pre-incubated with an excess of the A subunit (Table III), as already shown in Table I. The PP activity was further suppressed to 33% when the C subunit was pre-incubated with an excess of both the A- and B′δ-subunits (Table III), indicating that the A subunit is essential for the inhibitory effect of the B′δ subunit on the PP activity of CA. These results are consistent with the results that the B′δ subunit does not form a complex with the C subunit alone, but forms a heterotrimeric complex, CAB′δ, with the C subunit through the A subunit.

DISCUSSION

Previously (*11*), we proposed the subunit structure of major human erythrocyte PP2A to be CA based on the apparent molecular weights of the holoenzyme (104,000), the catalytic C subunit (32,000), and the A subunit (69,000). Furthermore, SDS-PAGE of purified CA showed the two Coomassie Blue-stained bands of the C- and A-subunits at a molar ratio of 1:1. In this paper, CA was dissociated into the C- and A-subunits by gel filtration on Sephacryl S-200 in the presence of 6 M urea, and then the dissociated C- and A-subunits were reassociated in the absence of urea. Titration of the C subunit with increasing amounts of the A subunit resulted in saturation of the A subunit at a molar ratio of 1:1 (Fig. 4). Mixing equimolar concentrations of the C- and A-subunits instantaneously formed an M_r=104,000 complex that produced two Coomassie Blue-stained bands for the 34-kDa C- and 63-kDa A-subunits at a molar ratio of 1:1 (Fig. 3), confirming that the subunit structure of the major PP2A (phosphatase IV in Ref. *11*) in human erythrocyte cytosol is comprised of a catalytic C subunit and an inactive A subunit.

The effects of an equimolar concentration of the A subunit on the PP- and PTP-activities of the C subunit varied with the substrate and assay conditions. Although the PP activities of the C subunit toward phosphorylase *a*, P-H1 histone, and P-H2B histone were suppressed by association with the A subunit under the conventional assay conditions, the P-H1 histone phosphatase activity was stimulated in the presence of 200 mM NaCl (Table I). The Mn^{2+}-dependent PTP activity toward P-Tyr-Glu copolymers was also enhanced about 2-fold by the association with the A subunit (Fig. 4). Similar opposite effects on the PP- and PTP-activities were also observed when CA was pre-incubated with NaF, which can react with metals in the enzyme (*13*). The catalytic C subunit of PP2A has been shown to be a metalloenzyme (*19, 20*). NaF completely inhibits the PP activity, but stimulates the Mn^{2+}-dependent PTP activity more than 10-fold (*13*). The opposite effects of the association with the A subunit on the PP- and PTP-activities of the C subunit may be attributable to the conformational change of the C subunit. The conformational change may hinder the substrate for the PP activity from binding to the catalytic dinuclear metal centers, but may facilitate the catalysis of the Mn^{2+}-dependent PTP activity by a mechanism different from that of the PP activity (*13*). These results indicate that the A subunit modulates the catalytic activity of the C subunit.

Using the same heparin-Sepharose column chromatography employed in this paper, CAB′δ highly purified from human erythrocyte cytosol was also dissociated into CA and the B′δ subunit (*3, 14*). The mixing of CA and the B′δ subunit produced an M_r=175,000 complex that produced three Coomassie Blue-stained bands for the C-, A-, and B′δ-subunits in a 1:1:1 molar ratio (data not shown). However, the reconstructed complex showed enzymatic properties similar to those of CA rather than those of CAB′δ. Furthermore, the B′δ subunit isolated from purified CAB′δ had no inhibitory effect on the PP activity of CA toward P-H2B histone. Since the B′δ subunit isolated from partially purified CAB′δ inhibited the PP activity of purified CA, failure for the enzymic properties to be reconstituted from purified CAB′δ may be due to denaturation of the B′δ subunit during the isolation procedures. The dissociation and reassociation of CAB were achieved with a partially purified preparation from pig heart, but were not successful with a purified preparation (*6*). The 56-kDa B subunit isolated from pig heart is a heat-labile protein that associates with CA to produce a tightly bound complex, CAB, and inhibits the PP activity toward phosphorylase *a* and glycogen synthase *b* significantly, but inhibits the activity toward phosphorylated histones in the absence of Mg^{2+} only slightly (*6*). The B subunit does not associate directly with the catalytic C subunit and does not inhibit the PP activities of the C subunit (*6*). In this paper, the B′δ subunit isolated

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from CAB′$_\delta$ partially purified from human erythrocyte cytosol also did not associate directly with the C subunit and did not inhibit the PP activity (Table III). The A subunit is essential for B and B′$_\delta$ to regulate the PP- and PTP-activities of the C subunit.

Comparison of the molecular activities of CA and CAB′$_\delta$ suggests that the B′$_\delta$ subunit suppresses the PP activities of CA toward P-spectrin, phosphorylase *a*, P-H1 histone, and P-H2B histone (*3*). The inhibitory effect of the B′$_\delta$ subunit on the PP activities of CA was confirmed by the reconstitution experiments presented in this paper.

It has been shown that the A subunit consists of 15 imperfect repeats and has a rod-like shape (*21, 22*). Subsequent deletion analysis mapped the sites of interaction with the C subunit to the carboxyl-terminal half (repeats 11-15) and the sites of interaction with the B-, B′-, and PR59/PR72-subunits, SV 40 small T antigen, and polyoma virus small- and middle-T antigens to the amino-terminal half (repeats 1-10) (*8, 23-25*). Using site-directed mutagenesis of the A subunit, the yeast two-hybrid system, and native gel analysis of mutant A subunits synthesized *in vitro*, it was demonstrated that the binding of the C subunit to the A subunit stabilizes the binding of the B- and B′-subunits to the amino-terminal half of the A subunit (*8, 23-25*). However, cross-linking of purified bovine CAB with 1,6-bismaleimidohexane, which reacts with sulfhydryl groups, produces a scarcely detectable cross-linked form of BC in contrast to the clearly detectable cross-linked forms of CAB, CA, and AB (*24*). It is not clear at present whether the effect of the B′$_\delta$ subunit on the PP- and PTP-activities of CA results from a direct interaction between the B′$_\delta$- and C-subunits or from a B′$_\delta$-induced indirect conformational change in the C subunit through the A subunit. These structural findings are consistent with the results of reconstruction experiments with pig heart CAB (*6*) and human erythrocyte CAB′$_\delta$ presented in this paper. These results support the notion that the A subunit not only functions as a scaffold protein to construct a heterotrimeric form of PP2A, but also functions as an allosteric modulator of the catalytic activity of the C subunit by transmitting the regulatory signals from the third subunits such as the B-, B′-, and PR59/PR72-subunits, and the viral antigens.

Molecular cloning of the B′$_\delta$ subunit revealed that in addition to the central 400-amino-acid sequence common to the B′ family, the predicted primary structure of the subunit contains a unique PQ repeat in the N terminal unique region, a bipartite motif of a putative nuclear localization signal, and an SH3 accessible proline-rich sequence in the C terminal unique region (*26*). The binding of the specific proteins to these unique regions may target the heterotrimeric form of PP2A to a certain locus such as the nucleus of a cell, and/or may modulate the PP- or PTP-activities of the C subunit through the A subunit.

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