J. Biochem. 122, 178-187 (1997)

Tissue and Subcellular Distributions, and Characterization of Rat Brain Protein Phosphatase 2A Containing a 72-kDa δ/B″ Subunit[1]

Terumasa Nagase,[2] Takehiko Murakami,[2] Hideto Nozaki,[2] Rintaro Inoue,[3] Yasumasa Nishito, Osamu Tanabe, Hirofumi Usui, and Masao Takeda[4]

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

Received for publication, February 24, 1997

A 74-kDa δ/B″ subunit was isolated by heparin-Sepharose column chromatography from human erythrocyte protein phosphatase 2A (PP2A) consisting of a 34-kDa catalytic subunit (α/C) and 63- and 74-kDa regulatory subunits (β/A and δ/B″) in a ratio of 1:1:1. The purified δ/B″ was used as an immunogen in mice, to prepare specific antisera against δ/B″. Immunoblot analyses with the antisera detected an immunoreactive 72-kDa protein in the cytosol from various rat tissues including erythrocytes, brain, lung, testis, adrenal gland, heart, spleen, kidney, and liver. The 72-kDa protein was highly abundant in brain and was distributed evenly in cerebral cortex, cerebellum, and brain stem. The 72-kDa protein was also detected in mitochondria and microsome fractions. An immunoreactive 68-kDa protein was detected mainly in nuclear and microsome fractions. The 72-kDa protein from rat brain cytosol copurified with phosphorylated H2B histone phosphatase activity during successive chromatographies on DEAE-Toyopearl, AH-Sepharose, Sephadex G-150, H1 histone-Toyopearl, TSK DEAE-5PW, protamine-Toyopearl, and TSK G3000SW columns. The purified enzyme migrated as a single protein band on nondenaturing PAGE and as three protein bands of 34, 63, and 72 kDa in a ratio of 1:1:1 on SDS-PAGE. The molecular weight of the enzyme was estimated to be 170,000 from the $s_{20,w}$ value of 7.2±0.3 S and the Stokes radius of 5.5±0.1 nm. The rat brain enzyme was classified as PP2A, based on the following properties; (1) an IC_{50} for okadaic acid of 10^{-9} M; (2) its preferential dephosphorylation of the α subunit of phosphorylase kinase; (3) its insensitivity to protein inhibitor 2; and (4) its heterotrimeric subunit structure. The K_m value and the molecular activity of the enzyme for phosphorylated H2B histone were 72.3±0.3 μM and 192±2 mol P_i released/min/mol enzyme, respectively, and were comparable to those of human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″). The 72-kDa subunit in the purified rat brain PP2A was phosphorylated *in vitro* by cAMP-dependent protein kinase.

Key words: phosphorylation, protein phosphatase 2A, rat brain, regulatory subunit 72-kDa δ/B″, tissue and subcellular distributions.

Protein phosphatase 2A (PP2A), one of the four major protein serine/threonine phosphatases, is found in all eukaryotic cells (*1*) and plays a crucial role in the regulation of many cellular events including metabolism, the cell cycle, cell proliferation, replication, transcription, translation, and viral transformation (*1-3*). Holoenzymes of PP2A which have molecular weights of more than 100,000 have been purified to near homogeneity from various mammalian tissues (*1*). These holoenzymes have either a heterodimeric or a heterotrimeric subunit structure. The heterodimeric structure is composed of a 32-41-kDa catalytic subunit α/C complexed to a 60-69-kDa subunit β/A. The dimeric structure $\alpha_1\beta_1$/CA is common to all PP2A holoenzymes. The heterotrimeric structure contains an additional subunit of either 51-58 kDa (γ/B), 54 kDa (B′), 72 kDa (PR72), or 74 kDa (δ/B″).

Previously, we purified from human erythrocyte cytosol three forms of PP2A, whose subunit structures are $\alpha_1\beta_1$/CA, $\alpha_1\beta_1\gamma_1$/CAB, and $\alpha_1\beta_1\delta_1$/CAB″ where α/C is a 34-kDa catalytic subunit and β/A, γ/B, and δ/B″ are 63-, 53-, and 74-kDa subunits, respectively (*4*). The 74-kDa δ/B″-containing PP2A ($\alpha_1\beta_1\delta_1$/CAB″) was most similar to $PP2A_0$ (CAB′) in elution position from a DEAE column, in heterotrimeric subunit structures, and in responses to polycations and heparin (*4-7*). In spite of the similarities, the 74-kDa δ/B″-containing PP2A was obviously different from $PP2A_0$ in the molecular mass of the third subunits, 74-kDa δ/B″

[1] This work was supported in part by Grants-in-Aid for Cancer Research and Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (1990-1996).

Present addresses: [2] Third Department of Internal Medicine, National Defense Medical College, Namiki, Tokorozawa, Saitama 359; [3] Department of Internal Medicine (II), Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734.

[4] To whom correspondence should be addressed. Tel: +81-82-257-5135, Fax: +81-82-257-5088, E-mail: mtakeda@mcai.med.hiroshima-u.ac.jp

Abbreviations: A-kinase, cAMP-dependent protein kinase; IC_{50}, 50% inhibitory concentration; PCS_M, polycation-stimulated protein phosphatase M; P-H2B histone, H2B histone phosphorylated by A-kinase; PMSF, phenylmethylsulfonyl fluoride; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PVDF, polyvinylidene difluoride; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone.

and 54-kDa B′. However, the recent molecular cloning of δ/B″ (*8*) and B′ (*9–13*) revealed that δ/B″ and B′ have a very similar sequence of more than 400 amino acid residues in the central regions of the molecules with more divergent N- and C-terminal sequences. Thus, δ/B″ and B′ belong to the same family. PCR and screening of cDNA libraries from rabbit and human have identified at least five isoforms of mammalian B′ and δ/B″ family encoded by distinct genes and seven possible splicing variants derived from three of the five genes. The predicted primary structure of δ/B″ is identical with that of human B56δ (*13*), except for a 32-amino acid insertion beginning at residue 84 of B56δ. The δ/B″ subunit is also nearly identical with rabbit B′γ (*9*), which contains the same 32-amino acid insertion and a unique N-terminal 4-residue sequence replacing an N-terminal 20-residue sequence of δ/B″. Comparison of the nucleotide sequences of the cDNAs for human δ/B″, human B56δ, and rabbit B′γ suggests that they may be produced by alternative splicing. From a human cerebral cortex cDNA library, we also isolated a cDNA clone for a variant of δ/B″ which contains the 32-amino acid insertion (unpublished observation). Among the isoforms of the B′ family, δ/B″, B56δ, and B′γ are the only isoforms whose apparent molecular masses (66–70 kDa) based on the cDNA sequence are larger than that of the 63-kDa β/A subunit. Other B′ isoforms have apparent molecular masses of 51–61 kDa.

In this paper, using specific antisera against the 74-kDa δ/B″ subunit, a heterotrimeric form of PP2A was detected in rat brain cytosol and shown to contain an immunoreactive 72-kDa subunit in addition to the 34- and 63-kDa subunits ($\alpha_1\beta_1$/CA). This form of PP2A was purified, characterized, and shown to correspond to the human erythrocyte 74-kDa δ/B″-containing PP2A. The 72-kDa δ/B″ was distributed widely in various rat tissues with the highest level in brain and the lowest levels in intestine and skeletal muscle. The ubiquitous distribution of the 72-kDa δ/B″ is consistent with the ubiquitous expression of the δ/B″ mRNA of about 2.9 kb in rat tissues (*8*). In subcellular fractionation analysis using rat brain, the 72-kDa δ/B″ subunit was detected not only in cytosol, but also in mitochondria and microsome fractions, and an immunoreactive 68-kDa protein was detected in the nuclear fraction.

EXPERIMENTAL PROCEDURES

Materials—Calf thymus H2B histone and H1 histone, rabbit skeletal muscle phosphorylase *b* and phosphorylase kinase, pig heart A-kinase, and its catalytic subunits were prepared as described previously (*4*). PP1 catalytic subunit was prepared from rabbit skeletal muscle as described by Resink *et al.* (*14*). Inhibitor 2 was prepared from rabbit skeletal muscle as described by Foulkes and Cohen (*15*). [γ-^{32}P]ATP was obtained from ICN Biomedicals. ^{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]phosphorylase kinase (4.2 nmol of bound ^{32}P/mg phosphorylase kinase, 0.80 mol of ^{32}P in each α subunit and 0.54 mol of ^{32}P in each β subunit) were prepared as described previously (*4*). Sepharose 4B, AH-Sepharose 4B, and heparin-Sepharose 6B were purchased from Pharmacia. Toyopearl (HW-65, superfine), DEAE-Toyopearl, TSK DEAE-5PW, and TSK G3000SW columns were obtained from Tosoh. CNBr-activated Toyopearl, H1 histone-Toyopearl, and protamine-Toyopearl were prepared as described previously (*16*). Ribi adjuvant system (52-0177-00, trehalose dimycolate containing an oil-in-water emulsion system) was from Ribi Immunochem Research. Horseradish peroxidase-labeled goat anti-mouse IgG was from Kirkegaard and Perry Laboratories. Enhanced chemiluminescence system was from Amersham. Bovine serum albumin, horse heart cytochrome *c*, bovine liver catalase, yeast alcohol dehydrogenase, PMSF, TPCK, human γ-globulin, ovalbumin, and salmon protamine were obtained from Sigma. Horse spleen ferritin was purchased from Boehringer-Mannheim. Leupeptin, antipain, and pepstatin were from the Peptide Institute. Silver stain kit was purchased from Daiichi Pure Chemicals. Colloidal gold total protein stain reagent (blotting grade) was obtained from Bio-Rad Laboratories. Okadaic acid was purchased from Wako Pure Chemical Industries. Other chemicals were obtained from various commercial sources.

Buffer Solutions—All the buffer solutions contained 0.5 mM DTT, 0.005% (v/v) Triton X-100, and protease inhibitors including 0.1 mM PMSF, 1 mM benzamidine, 0.5 mg/liter pepstatin, 0.5 mg/liter antipain, 1 mg/liter leupeptin, and 10 mg/liter TPCK. In addition to the above constituents, Buffer A contained 10 mM imidazole, pH 6.5 and 10% (v/v) glycerol; Buffer B contained 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 0.25 M sucrose; Buffer C contained 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 10% glycerol; Buffer D contained 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 10% glycerol; and Buffer E contained 20 mM HEPES, pH 7.0, 1 mM EDTA, and 10% glycerol.

Enzyme Assays—Phosphatase activity was measured in a reaction mixture (50 μl) containing 100 μM ^{32}P-H2B histone, 50 mM Tris-HCl, pH 7.4, 50 mM magnesium acetate, 0.5 mM DTT, 0.05% Triton X-100, and an enzyme fraction (standard assay conditions). The reaction was started by the addition of the substrate, carried out for 10 min at 30°C, and terminated by the addition of 1 ml of 5 mM silicotungstic acid in 2.5 mM H_2SO_4. The [^{32}P]P_i released was measured as described previously (*4*). The substrate concentration represents the concentration of ^{32}P bound to the substrate protein. One unit of enzyme was defined as the amount of enzyme which catalyzed the release of 1 nmol of [^{32}P]P_i per min.

Isolation of 74-kDa δ/B″ from a PP2A Holoenzyme—Unless otherwise stated, all procedures were carried out at 0–4°C. PP2A, which has a subunit structure of $\alpha_1\beta_1\delta_1$/CAB″, was purified from human erythrocytes as described previously (*4*). PP2A ($\alpha_1\beta_1\delta_1$/CAB″) was dialyzed against 500 ml of Buffer A and 38.8 units, 63.4 μg protein, 1.1 ml were applied to a heparin-Sepharose 6B column (2×0.8 cm) equilibrated with Buffer A. The column was washed with 20 ml of the same buffer, then the 74-kDa δ/B″ was eluted with a 60 ml linear 0–0.7 M NaCl gradient in Buffer A. The flow rate was 8 ml/h and 1.0 ml fractions were collected in polypropylene tubes. Proteins in each fraction were detected by SDS-PAGE (10% gel) followed by silver staining. $\alpha_1\beta_1$/CA was eluted at 0.04 M NaCl and the 74-kDa δ/B″ was eluted at 0.4 M NaCl. To the 74-kDa δ/B″ was added 100% (w/v) trichloroacetic acid to a final concentration of 12%. After standing on ice for 1 h, the precipitate was collected by centrifugation for 30 min at 16,000×*g*, and washed three times with ice-cold acetone.

From a liter of packed human erythrocytes, 170 μg of 74-kDa δ/B″ was obtained. The dried δ/B″ preparation was dissolved in 0.1% (w/v) SDS containing 10 mM Tris-HCl, pH 7.5 at a concentration of 5-10 μg/100 μl.

Preparation of Anti-74-kDa δ/B″ Antisera—The purified 74-kDa δ/B″ (5-10 μg) in 100 μl of 0.1% SDS containing 10 mM Tris-HCl, pH 7.5, was vortexed with 100 μl of Ribi adjuvant system and injected intraperitoneally into two female BALB/c mice (6 weeks old). Three weeks later the mice were given a booster injection of the same amount of the protein with Ribi adjuvant, followed by two additional booster injections every three weeks. The mice were bled one week after each booster injection and the sera were tested for the presence of antibodies by immunoblotting against the purified human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″). Four separate injections of the 74-kDa δ/B″ into a mouse produced a high titer (1:2,000-1:8,000 dilution) of anti-74-kDa δ/B″ antiserum. The whole sera were taken one week after the final booster injection and stored at −80°C.

Preparation of Tissue Samples—Male Wistar albino rats (200-250 g) were starved overnight and killed by decapitation. Brains, livers, skeletal muscles, testes, spleens, intestines, hearts, lungs, kidneys, and adrenals were removed quickly. Unless otherwise stated, all manipulations were carried out at 0-4°C. Brains from 3 rats were dissected (*17*) into cerebellum (0.97 g), cerebral cortex including hippocampus (3.93 g), and brain stem containing midbrain, hypothalamus, striatum, and medulla oblongata (1.80 g). Tissues were chopped into small pieces, and homogenized in 5 volumes (v/w) of ice-cold Buffer B with a motor-driven Teflon-glass homogenizer (20 strokes). The homogenate was filtered through 4 layers of gauze and the filtrate was centrifuged for 10 min at 12,500×g. The supernatant was centrifuged for 90 min at 113,000×g and the resulting supernatant was used as a cytosolic fraction. The pellets from the first and second centrifugations were suspended in 4 volumes and one volume (to the original wet weight of tissues), respectively, of Buffer B with a motor-driven Teflon-glass homogenizer (10 strokes) and used as particulate and microsome fractions. Some of the tissues were quickly frozen in liquid nitrogen and stored at −80°C (Fig. 1C). The frozen tissues were dipped into liquid nitrogen and pulverized in a precooled metallic mortar with a pestle (Plattner's diamond mortar and pestle set, Fisher Scientific) by hitting the pestle 5-10 times with a hammer. Cytosol was prepared from tissue powder as described above.

After decapitation, 10 ml of blood from two rats were collected into a centrifuge tube containing 8 ml of 3.8% (w/v) citric acid. Erythrocytes were collected by centrifugation for 10 min at 1,000×g at 4°C and the white-cell layer was removed by aspiration. Erythrocytes were washed three times with 20-25 ml of saline and lysed in 18 ml of 20 mM Tris-HCl, pH 7.4, 0.5 mM DTT, 1 mM EDTA, and protease inhibitors using a Dounce homogenizer (tight-fitting, 20 strokes). Hemolysates were centrifuged for 2 h at 105,000×g and the supernatant was used as cytosol.

Tissue Fractionation—Brains from three starved male Wistar albino rats were homogenized in 9 volumes (v/w) of Buffer B containing 0.32 M sucrose with a motor-driven Teflon-glass homogenizer (10 strokes) and fractionated into nuclear, mitochondria, microsome, and soluble fractions (*18*). The nuclear fraction was further purified by homogenization in Buffer B containing 2.0 M sucrose followed by centrifugation for 75 min at 105,000×g (*19*). The pellet was suspended in Buffer B and used as a nuclear fraction.

Removal of Hemoglobin from Erythrocyte Cytosol—Fifteen milliliters of rat erythrocyte cytosol was applied to a DEAE-Sephadex column (5×1 cm) equilibrated with 0.05 M NaCl in Buffer C at a flow rate of 30 ml/h. The column was washed with 10 ml of 0.05 M NaCl in Buffer C, and proteins containing P-H2B histone phosphatase activity were eluted with 0.5 M NaCl in Buffer C at a flow rate of 10 ml/h. The eluate was passed through a continuous-flow countercurrent dialyzing apparatus (Biomed. Instruments, model D-1, flow rate 50 ml/h) for removal of NaCl by dialysis against Buffer C.

Purification of the Rat Brain Protein Phosphatase Containing a 72-kDa Subunit—All procedures were carried out at 0-4°C. Cytosol was prepared from 101 g of pulverized rat brain as described above. The cytosol fraction (17,600 units, 2,200 mg protein, 305 ml) was applied to a DEAE-Toyopearl column (30×3.2 cm) equilibrated with 0.03 M NaCl in Buffer C at a flow rate of 200 ml/h. The column was washed with 500 ml of Buffer C, and protein phosphatases were eluted with a 3,400 ml linear 0.03-0.25 M NaCl gradient in Buffer C. The flow rate was 200 ml/h and 18 ml fractions were collected. Seven activity fractions which were eluted at the NaCl concentration of 0.06, 0.10, 0.11, 0.12, 0.13, 0.16, and 0.19 M, respectively, were numbered from I to VII in the order of elution, as shown in Fig. 3. Since only peak I fractions contained the immunoreactive 72-kDa protein (Fig. 3), peak I fractions indicated by a solid bar in Fig. 3 were pooled, and tentatively designated as the rat brain protein phosphatase. The pooled peak I fractions (1,256 units, 82.5 mg protein, 345 ml) were loaded onto an AH-Sepharose 4B column (15×1.6 cm) equilibrated with 0.1 M NaCl in Buffer C. The column was washed with 150 ml of the same buffer, and the enzyme was eluted with a 600 ml linear 0.1-0.5 M NaCl gradient in Buffer C. The flow rate was 45 ml/h and 6 ml fractions were collected. The active peak fractions eluted at 0.21 M NaCl were pooled and concentrated to 9.2 ml in an Amicon ultrafiltration cell equipped with a YM-10 membrane. The AH-Sepharose fraction (842 units, 50.6 mg protein, 9.2 ml) was subjected to gel filtration on a Sephadex G-150 column (80×2.5 cm) equilibrated with Buffer D. Gel filtration was performed upward with Buffer D at a flow rate of 20 ml/h. Fractions of 4 ml each were collected. The active fractions were pooled. The pooled fraction (438 units, 19.3 mg protein, 38 ml) was applied to an H1 histone-Toyopearl column (5.0×1.6 cm) equilibrated with Buffer D. The column was washed with 150 ml of the same buffer, and the enzyme was eluted with a 200 ml linear 0-0.12 M NaCl gradient in Buffer D. The flow rate was 20 ml/h and 3 ml fractions were collected. The active fractions eluting at 0.04 M NaCl were pooled. The pooled fraction (347 units, 3.73 mg protein, 23 ml) was loaded onto a TSK DEAE-5PW column (15×2.15 cm) equilibrated with 0.02 M NaCl in Buffer C, using a Tosoh HPLC system. The column was washed with 10 ml of the same buffer and the enzyme was eluted with a 500 ml linear 0.02-0.12 M NaCl gradient in Buffer C. The flow rate was 2.5 ml/min and 4 ml fractions were collected. The active fractions eluted at 0.085 M NaCl were collected. The collected fraction (209

units, 0.62 mg protein, 19.5 ml) was applied to a protamine-Toyopearl column (6.4×1.0 cm) equilibrated with 0.1 M NaCl in Buffer C. The column was washed with 15 ml of the same buffer and the enzyme was eluted with a 150 ml linear 0.1-0.7 M NaCl gradient in Buffer C. The flow rate was 10 ml/h and 2 ml fractions were collected. The enzyme was eluted at 0.43 M NaCl as a single peak. The active fractions were pooled and concentrated to 2.6 ml using an Amicon YM-10 membrane. The concentrated fraction (183 units, 0.21 mg protein, 2.6 ml) was subjected to gel filtration on a TSK G3000SW column (60×2.15 cm) equilibrated with 0.3 M NaCl in Buffer D, using a Tosoh HPLC system. The flow rate was 1.0 ml/min and 1.0 ml fractions were collected. The active fractions were pooled and stored at −80°C.

A summary of the purification is presented in Table I.

Gel Electrophoresis—Nondenaturing PAGE was performed in 6.5% polyacrylamide disc gels with System I as described by Gabriel (*20*) with a slight modification as described previously (*16*). SDS-PAGE was performed as described previously (*4*), with a stacking 3% gel and a separating 10% gel. One-third volume of 40 mM Tris-HCl, pH 7.8, 12% SDS, 20% glycerol, 10 mM DTT, and 0.07% (w/v) bromophenol blue (SDS-sample buffer), were added to the samples, which were then heated in a boiling water bath for 3 min. One-sixth volume of freshly prepared 0.63 M iodoacetamide was added to the samples, which were then subjected to SDS-PAGE. Protein was detected by either Coomassie Blue staining (*21*) or silver staining (*22*). Molecular mass standards were phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), a heavy chain of human γ-globulin (50 kDa), and ovalbumin (43 kDa).

Immunoblotting—After SDS-PAGE, proteins in gels were transferred electrophoretically to PVDF membranes in 25 mM Tris, 192 mM glycine, and 0.1% SDS at 2 mA/cm² for 1 h. The PVDF membranes were then blocked with 100 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, 0.1% (v/v) Tween 20 (PBST) containing 5% (w/v) skimmed milk for 1 h. The membranes were incubated for 1 h with anti-74 kDa δ/B″ antisera diluted (2-8×10³-fold) in PBST, and incubated for 1 h with horseradish peroxidase-labeled goat anti-mouse IgG diluted (1×10³-fold) in PBST. The detection of immunoreactive bands was performed by using an enhanced chemiluminescence system as directed by the manufacturer. Protein bands on PVDF membranes were detected by use of a colloidal gold system.

Dephosphorylation of the α and β Subunits of Phosphorylase Kinase—Dephosphorylation of [^{32}P]phosphorylase kinase was performed at 30°C in a reaction mixture (30 μl) containing 1 μM [^{32}P]phosphorylase kinase, 50 mM Tris-HCl, pH 7.4, 2 mM $MnCl_2$, 0.5 mM DTT, 0.05% Triton X-100, and protein phosphatase preparations (46 milliunits/ml with phosphorylase kinase as the substrate) for 5 and 10 min. The α and β subunits of phosphorylase kinase were separated by 7% SDS-PAGE and stained with Coomassie Blue. Gels were dried and the bands corresponding to the α and β subunits were excised and their radioactivity was determined.

Phosphorylation of Rat Brain PP2A by A-Kinase—A hundred nanograms of rat brain PP2A or human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) was incubated for 10 min at 30°C in a 30 μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, and 10 μM [γ-^{32}P]ATP (1,800 cpm/pmol) with catalytic subunits (30 ng, 500 units/mg) of A-kinase. Reactions were stopped by adding 10 μl of SDS-sample buffer. After heating in a boiling water bath for 3 min, 30 μl of sample was applied to SDS-PAGE.

Determinations—The molecular weight of rat brain PP2A was determined by the method of Siegel and Monty (*23*) from the $s_{20,w}$ value and the Stokes radius. The $s_{20,w}$ value was determined by sucrose density gradient centrifugation as described previously (*24*) with catalase (11.3S), alcohol dehydrogenase (7.4S), and cytochrome *c* (1.9S) as standards. The Stokes radius was estimated by gel filtration on a Sephadex G-150 column with ferritin (7.9 nm), human γ-globulin (5.55 nm), bovine serum albumin (3.5 nm), ovalbumin (2.73 nm), and cytochrome *c* (1.7 nm) as standards under the same conditions as described for the purification of the rat brain protein phosphatase. Protein was determined by the method of Lowry *et al.* (*25*) or Bradford (*26*) with bovine albumin as a standard.

RESULTS

Tissue and Subcellular Distribution of the δ/B″ Subunit in Rat—The tissue distribution of the δ/B″ regulatory subunit of PP2A was examined using antisera against the δ/B″ subunit prepared in mice by intraperitoneal injections of the human erythrocyte 74-kDa δ/B″ subunit with an oil-in-water type Ribi adjuvant system. Antisera against the δ/B″ subunit thus obtained reacted specifically with the 74-kDa δ/B″ subunit, but not with the 34-kDa α/C, the 63-kDa β/A (Fig. 1, B and C, and Fig. 4B), or the 53-kDa γ/B (data not shown) subunit of human erythrocyte PP2A. Immunoblot analyses of cytosolic proteins from various rat tissues with the antisera revealed that an immunoreactive 72-kDa protein band was detected in cytosol prepared from various rat tissues, including erythrocytes (Fig. 1A), brain, spleen, kidney, liver (Fig. 1B), lung, testis, adrenal gland, and heart (Fig. 1C). Little if any immunoreactive 72-kDa protein was detected in intestine and skeletal muscle (Fig. 1C). Since the immunoreactive 72-kDa protein was most abundant in brain cytosol (Fig. 1, B and C), the regional and subcellular distribution of the immunoreactive protein in brain was investigated (Fig. 2). The immunoreactive 72-kDa protein was evenly distributed in cytosol from cerebellum, cerebral cortex including hippocampus, and brain stem, which included the midbrain, hypothalamus, striatum, and medulla oblongata (Fig. 2). Distribution of the 72-kDa protein in particulate (12,500×*g* precipitate), microsome (12,500×*g* supernatant/113,000×*g* precipitate), and cytosol (12,500×*g* supernatant/113,000×*g* supernatant) fractions from each brain region showed that the cytosol contained the most 72-kDa protein and was the best source for the purification of the protein (Fig. 2). In the particulate and microsome fractions, an immunoreactive 68-kDa protein was detected in addition to the 72-kDa protein (Fig. 2). Cell fractionation revealed that the 68-kDa protein was distributed mainly in the nuclear fraction and the 72-kDa protein was detected in the mitochondrial fraction (Fig. 2).

Purification of the 72-kDa Protein—To investigate whether the 72-kDa protein is a regulatory subunit of PP2A, we subjected rat brain cytosol to DEAE-Toyopearl

column chromatography. Protein phosphatase activity was detected with P-H2B histone as the substrate (Fig. 3A) and the 72-kDa protein was identified by Western blotting (Fig. 3B). The 72-kDa protein was detected only in the first small peak of protein phosphatase activity which was eluted at the NaCl concentration of 0.06 M, and was designated tentatively as the rat brain protein phosphatase (Fig. 3B). The human erythrocyte PP2A, which has a subunit structure of $\alpha_1\beta_1\delta_1$/CAB″, was also eluted with 0.06–0.07 M NaCl from the same column (*4*). The protein phosphatase in the peak I fractions in Fig. 3 was pooled and purified by successive column chromatography on AH-Sepharose, Sephadex G-150, H1 histone-Toyopearl, TSK DEAE-5PW, protamine-Toyopearl, and TSK G3000SW. The final rat brain protein phosphatase preparation migrated as one main protein band on nondenaturing PAGE (Fig. 4A), had a specific activity of 1,295 units/mg protein and was purified 85-fold from the DEAE-Toyopearl preparation (Table I). The SDS-PAGE of the purified enzyme yielded three silver stained bands with molecular masses of 72 (δ), 63 (β), and 34 kDa (α) (Fig. 4B). Western blotting of the three subunits in the purified rat brain protein phosphatase showed that only the 72-kDa subunit reacted with antiserum against the 74-kDa δ/B″ of human erythrocyte PP2A (Fig. 4B). These results indicate that the immunoreactive 72-kDa protein in rat brain cytosol is indeed a subunit of a protein phosphatase.

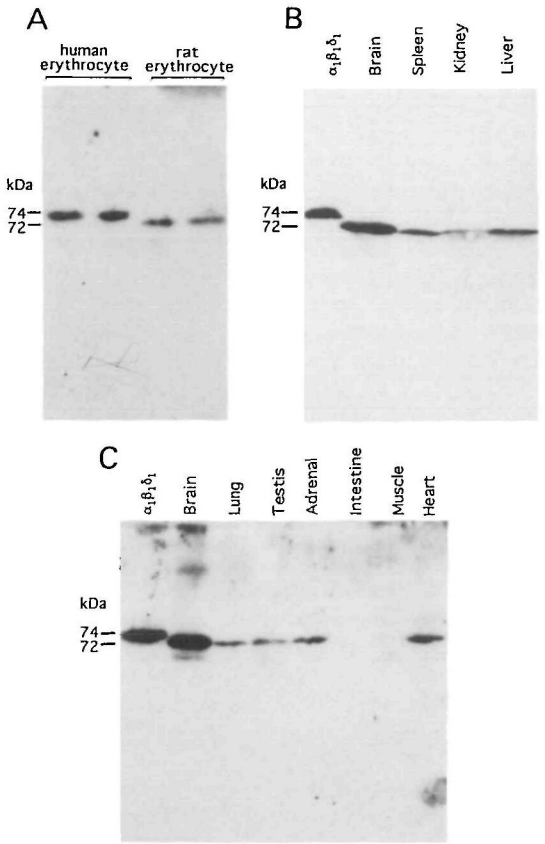


Fig. 1. **Immunoblotting of cytosolic proteins from various rat tissues with anti-74-kDa δ/B″ antisera.** SDS-PAGE followed by immunoblot analysis was performed on (panel A) hemoglobin-less cytosols (4.8 μg protein) from human and rat erythrocytes; (panel B) human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) (0.1 μg protein) and cytosol (120 μg protein) from the freshly homogenized rat tissues indicated; (panel C) human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) (15 ng protein) and cytosol (50 μg protein) from the pulverized rat tissues indicated, as described under "EXPERIMENTAL PROCEDURES."

Molecular Weight and Subunit Structure of the Rat Brain Protein Phosphatase—The Stokes radius of the purified protein phosphatase was determined by gel filtration on Sephadex G-150 to be 5.5±0.1 nm. The $s_{20,w}$ value of the purified enzyme was measured by sucrose density gradient centrifugation analysis to be 7.2±0.3 S. From these parameters, the apparent molecular weight of the purified enzyme was calculated to be 170,000. The SDS-PAGE of the purified enzyme yielded three Coomassie Blue stained bands with molecular masses of 72, 63, and 34 kDa (Fig. 5). Assuming that Coomassie Blue-staining of each subunit was equal, the molar ratio of α, β, and δ was estimated from a densitometric scan of the stained gel to be 1.07:1.00:1.02. The sum of the molecular masses of α, β, and δ was in good agreement with the molecular weight of 170,000 for the enzyme. From these results, the subunit structure of the rat brain protein phosphatase was determined to be $\alpha_1\beta_1\delta_1$.

Classification of the Rat Brain Protein Phosphatase as

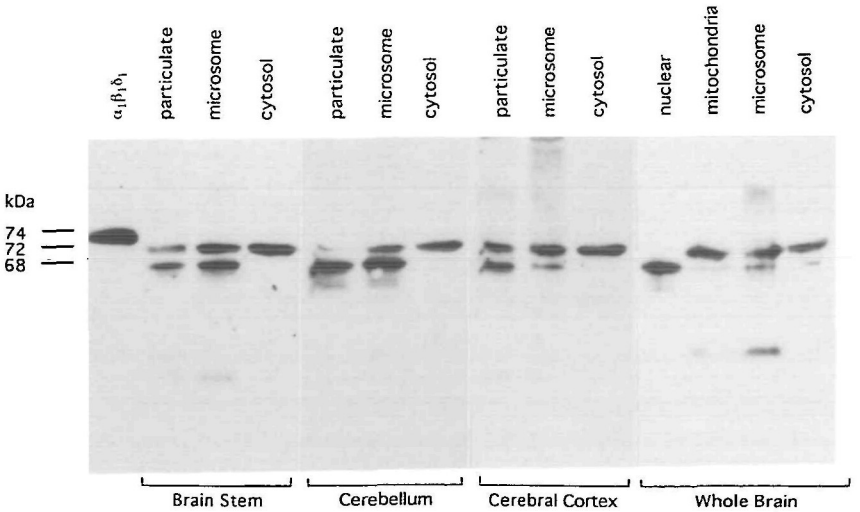
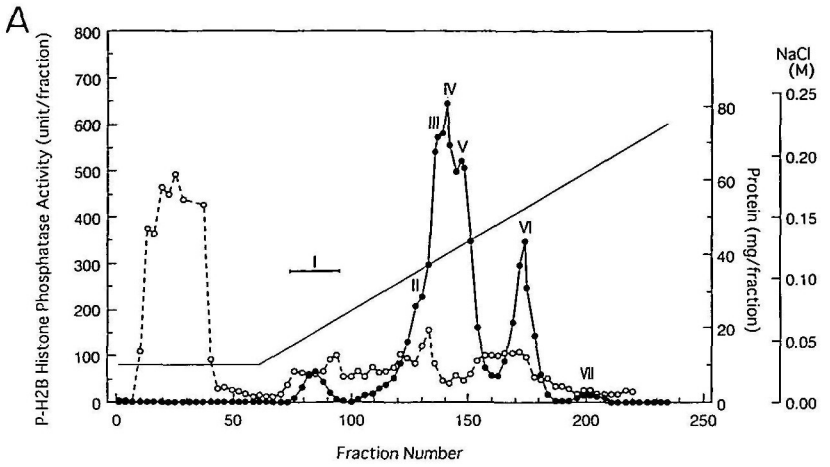


Fig. 2. **Regional and subcellular distribution of immunoreactive proteins with anti-74- kDa δ/B″ antisera in rat brain.** Dissection of rat brain, preparation of tissue fractions, and SDS-PAGE followed by immunoblot analysis of human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) (50 ng protein), each fraction (80 μg protein) from various regions in rat brain and nuclear, mitochondrial, microsome (80 μg protein each), and cytosol (50 μg protein) fractions from whole brain were performed as described under "EXPERIMENTAL PROCEDURES."



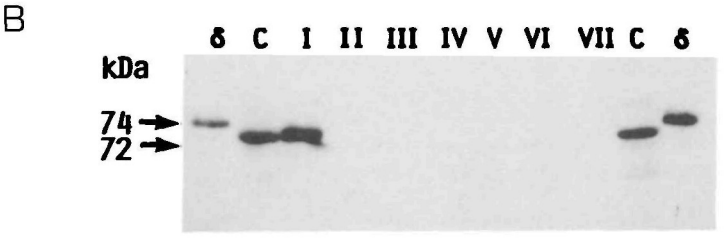


Fig. 3. **DEAE-Toyopearl elution profile of P-H2B histone phosphatase activity and an immunoreactive 72-kDa protein in rat brain cytosol.** (Panel A) Column chromatography, and assays for P-H2B histone phosphatase activity (●) and for protein (○) were carried out as described under "EXPERIMENTAL PROCEDURES." (Panel B) SDS-PAGE followed by immunoblot analysis was performed on human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) (50 ng protein) (δ), rat brain cytosol (50 μg protein) (C), and 30 μl each of fractions 85 (I), 127 (II), 137 (III), 141 (IV), 147 (V), 174 (VI), and 201 (VII), as described under "EXPERIMENTAL PROCEDURES."

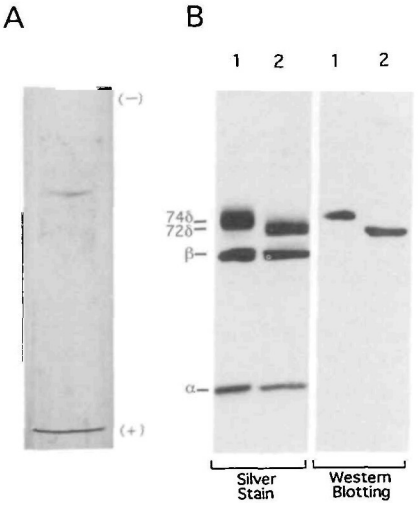


Fig. 4. **Nondenaturing and denaturing PAGE of the rat brain protein phosphatase.** (Panel A) Nondenaturing PAGE followed by Coomassie Blue staining of the rat brain protein phosphatase (2.5 μg protein). A piece of metal wire was inserted to mark the position of the dye front. (Panel B) SDS-PAGE followed by silver staining or immunoblotting with anti-74-kDa δ/B″ antisera of (lane 1) human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) (0.1 μg protein) and (lane 2) the rat brain protein phosphatase (0.1 μg protein).

TABLE I. **Purification of the rat brain protein phosphatase.** The enzyme was prepared from 101 g of rat brain. Purification and assay procedures are described under "EXPERIMENTAL PROCEDURES."

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Cytosol	2,200	17,600	8.0		
DEAE-Toyopearl (Peak I)	82.5	1,256	15.2	100	1
AH-Sepharose	50.6	842	16.6	67	1.1
Sephadex G-150	19.3	438	22.7	35	1.5
H1 histone-Toyopearl	3.73	347	93.0	28	6.1
TSK DEAE-5PW	0.62	209	337	17	22
Protamine-Toyopearl	0.21	183	871	15	57
TSK G3000SW	0.088	114	1,295	9	85

PP2A—The purified rat brain phosphatase was strongly inhibited by okadaic acid with an IC_{50} of 10^{-9} M (Fig. 6). The sensitivity of the rat brain enzyme to okadaic acid was comparable to that of human erythrocyte PP2A, whose subunit structure is $\alpha_1\beta_1\delta_1$/CAB″ (Fig. 6). Under the same conditions, the IC_{50} of okadaic acid for the inhibition of rabbit skeletal muscle PP1 was 2×10^{-8} M (Fig. 6). The purified rat brain phosphatase preferentially dephosphorylated the α subunit of phosphorylase kinase which was phosphorylated at both the α and β subunits by A-kinase under conditions in which the β subunit was preferentially dephosphorylated by PP1 (Fig. 7A). Furthermore, the phosphorylase phosphatase activity of the purified rat brain phosphatase was not affected by inhibitor 2 under conditions in which the activity of PP1 was strongly inhibited (Fig. 7B). These results indicate that the purified rat brain phosphatase can be classified as a heterotrimeric PP2A whose 72-kDa subunit δ is immunoreactive with antisera against the 74-kDa δ/B″ of human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″).

Kinetic Properties—The K_m value for P-H2B histone of the rat brain protein phosphatase was determined to be 72.3 ± 0.3 μM. The molecular activity for P-H2B histone of the enzyme was calculated to be 192 ± 2 mol P_i released/min/mol enzyme. These values are comparable to those of

human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) whose K_m and molecular activity are 63.9±9.9 μM and 121±10 mol P_i released/min/mol enzyme, respectively (*4*).

Phosphorylation of the 72-kDa Subunit by A-Kinase—Since the 74-kDa δ/B″ in human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) is phosphorylated *in vitro* by A-kinase (*7*), *in vitro*-phosphorylation of the 72-kDa δ in rat brain protein phosphatase ($\alpha_1\beta_1\delta_1$) by A-kinase was investigated. The purified rat brain protein phosphatase and human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) were separately incubated with a catalytic subunit of A-kinase and [γ-^{32}P]ATP for 10 min at 30°C, and the phosphorylation of these subunits was analyzed by SDS-PAGE, followed by autoradiography (Fig. 8). The 72-kDa δ was indeed phosphorylated by A-kinase under conditions in which the 74-kDa δ was also phosphorylated. No phosphorylation of the 34- and 63-kDa subunits was observed. Autophosphorylation of the catalytic subunit of A-kinase was also detected. A minor radioactive band which migrated more slowly than the catalytic subunit was a contaminant derived from the A-kinase preparation.

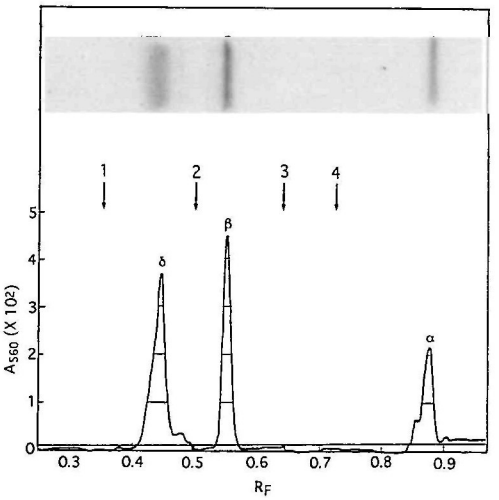


Fig. 5. **SDS-PAGE of the rat brain protein phosphatase.** SDS-PAGE of the rat brain protein phosphatase (0.2 μg protein) followed by Coomassie Blue staining was carried out as described under "EXPERIMENTAL PROCEDURES." The stained gel was scanned at 560 nm with a Gelman DCD-16 digital computing densitometer. Numbered arrows indicate phosphorylase (1), bovine serum albumin (2), heavy chain of human γ-globulin (3), and ovalbumin (4).

DISCUSSION

In this paper, the 74-kDa δ/B″ subunit was isolated by heparin-Sepharose column chromatography of a purified human erythrocyte $\alpha_1\beta_1\delta_1$/CAB″. Because the dissociated 74-kDa δ/B″ showed a propensity to bind to glass surfaces, it was kept in plastic (polypropylene) tubes in a 0.005% Triton X-100-containing buffer. Using the isolated 74-kDa δ/B″ as the immunogen in mice, we obtained an antiserum specific to 74-kDa δ/B″. This antiserum did not react with α/C, β/A, and γ/B subunits of human erythrocyte PP2A. A tissue survey of proteins immunoreactive to the antiserum revealed that only 72- and 68-kDa proteins (Fig. 2)

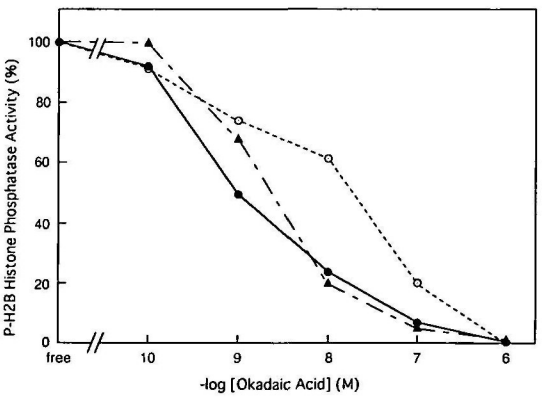


Fig. 6. **Effect of okadaic acid on the rat brain protein phosphatase.** P-H2B histone phosphatase activity was measured with the rat brain protein phosphatase ($\alpha_1\beta_1\delta_1$, 0.9 unit/ml) (●), human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) (1.1 units/ml) (▲), and the catalytic subunit (0.4 unit/ml) (○) of rabbit skeletal muscle PP1 under the standard assay conditions, except that okadaic acid at the indicated concentration was included. The activity without okadaic acid was taken as 100.

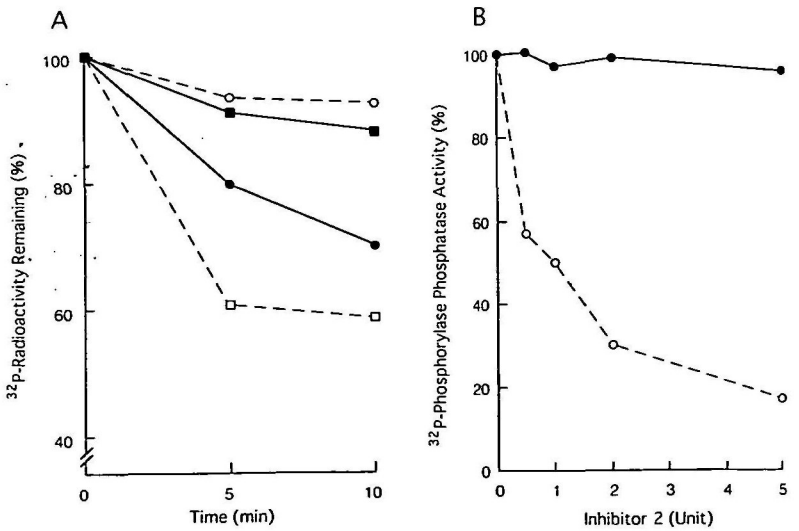


Fig. 7. **Classification of the rat brain protein phosphatase as PP2A.** (Panel A) Dephosphorylation of ^{32}P-labeled α (● and ○) and β (■ and □) subunits of phosphorylase kinase by the rat brain protein phosphatase (● and ■), and by the catalytic subunit of rabbit skeletal muscle PP1 (○ and □) were measured as described under "EXPERIMENTAL PROCEDURES." (Panel B) The purified rat brain phosphatase (0.17 unit/ml with phosphorylase *a* as the substrate) (●) or the catalytic subunit of PP1 (0.17 unit/ml with phosphorylase *a* as the substrate) (○) was preincubated at 30°C for 10 min with or without the indicated units of inhibitor 2 in 20 μl of 50 mM HEPES-NaOH, pH 7.4, 1.5 mM EDTA, 0.5 mM DTT, 3 mM $MnCl_2$, and 0.015% Triton X-100. This was followed by the addition of 10 μl of ^{32}P-labeled phosphorylase *a* (0.3 nmol) in 50 mM HEPES-NaOH, pH 7.4, and 0.5 mM DTT. The reaction mixture was further incubated at 30°C for 10 min. [^{32}P]P_i released was measured as described under "EXPERIMENTAL PROCEDURES." A unit of inhibitor 2 was defined as described (*15*).

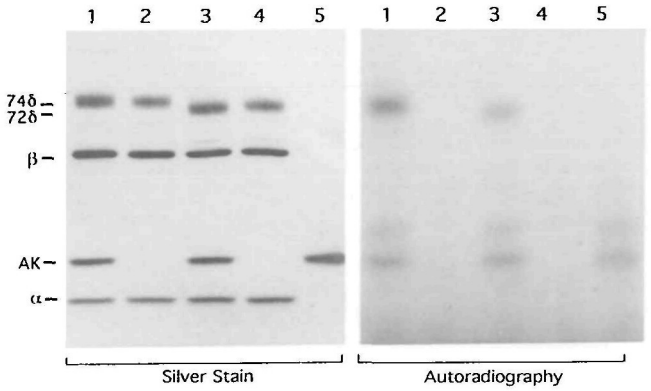


Fig. 8. **Phosphorylation of the 72-kDa subunit in rat brain PP2A by A-kinase.** Human erythrocyte PP2A ($\alpha_1\beta_1\delta_1$/CAB″) (lanes 1 and 2) and rat brain PP2A ($\alpha_1\beta_1\delta_1$) (lanes 3 and 4) were incubated with (lanes 1 and 3) or without (lanes 2 and 4) the catalytic subunit of A-kinase as described under "EXPERIMENTAL PROCEDURES." Lane 5 is a control without protein phosphatases. Protein bands were detected by silver staining and the phosphorylation was detected by autoradiography. Migration of α/C, β/A, and δ/B″ subunits of PP2A and the catalytic subunit of A-kinase (AK) is indicated.

reacted with the antiserum. Since δ/B″ and its possible alternatively spliced variants, human B56δ and rabbit B′γ, are the only B′ isoforms whose molecular masses are larger than that (63 kDa) of the A subunit among other B′ isoforms, the epitope of the antiserum could be an amino acid sequence in the variable N- or C-terminal regions which are unique to the large B′ isoforms.

The purification and characterization of the immunoreactive 72-kDa protein from rat brain cytosol revealed that the 72-kDa protein was indeed a subunit corresponding to human erythrocyte 74-kDa δ/B″ in a heterotrimeric PP2A ($\alpha_1\beta_1\delta_1$/CAB″). The differences in molecular mass of human and rat δ/B″ could be attributable to species differences, since all immunoreactive proteins in cytosols from all rat tissues tested had a molecular mass of 72 kDa. Reactivity of the rat 72-kDa δ/B″ to anti-74-kDa δ/B″ antisera was almost comparable to that to human 74-kDa δ/B″, indicating that the amino acid sequences immunoreactive to the antisera are well conserved between rat and human.

In rat brain nuclear fraction, an immunoreactive 68-kDa protein, but not 72-kDa δ/B″, was detected. The 68-kDa protein might be a splice variant of the 72-kDa δ/B″ subunit, or the 72- and 68-kDa proteins might be produced by different post-translational modifications, such as phosphorylation and processing. Indeed, several cDNAs for possible splicing variants of the δ/B″ subunit with different N-terminal sequences were identified from human and rabbit (*8, 9, 13*). Near the C-terminus of these variants, the bipartite motif of putative nuclear localization signal (KRTVETEAVQMLKDIKK) was identified (*27*). These findings suggest that this motif might be involved in nuclear localization of some δ/B″ isoforms, which may serve as nuclear targeting subunits of PP2A. Such putative nuclear localization signals were also found near the C-terminus of some of the B′ isoforms smaller than the 63-kDa β/A (*9, 10, 12*). The nuclear localization of overexpressed epitope-tagged B′ isoforms was detected by immunostaining (*10, 13*). Although most of the PP2A holoenzymes have been purified from cytosol of various mammalian tissues, a significant amount of PP2A is associated with nuclei; however, the precise subunit structure of nuclear PP2A has not been identified (*28-30*).

Cohen designated human erythrocyte 74-kDa δ as B″ (*1*). The nomenclature B″ was misused for PR72 in rabbit skeletal muscle PCS_M only because of the similarity of the molecular mass (*6*). Molecular cloning and tissue survey of PR72 (*31*) and δ/B″ (*8*) clearly showed that PR72 was distinct from 74-kDa δ/B″. To avoid confusion, this paper relies on the traditional names δ/B″ for the human erythrocyte 74-kDa subunit and the corresponding rat brain 72-kDa subunit, although B′ and δ/B″ belong to the same family, having very similar primary structures (more than 70% identity) of about 400 amino acid residues in the middle of the molecule (*8-13*).

Heterotrimeric forms consisting of two regulatory subunits of 63-69-kDa β/A and 51-58-kDa γ/B and a catalytic subunit of 34-41-kDa α/C, have been detected in brains from pigs (*32*), cattle (*6, 33, 34*), mice (*35*), and rats (*36*). Some of these have been immunochemically identified as mainly CABα and CAB′ (*6, 34, 36*). However, no heterotrimeric PP2A containing δ/B″ has been reported, probably for the following reasons: (1) $\alpha_1\beta_1\delta_1$/CAB″ was estimated to be less than 10% of total PP2A in rat brain cytosol from the data in Fig. 3A; (2) $\alpha_1\beta_1\delta_1$/CAB″ is a suppressed form, in which δ/B″ suppresses $\alpha_1\beta_1$/CA activity toward phosphorylated histones and phosphorylase *a* (*4*); (3) $\alpha_1\beta_1\delta_1$/CAB″ was eluted from DEAE-resins with 0.05-0.07 M NaCl, and some of the procedures for preparing PP2A holoenzyme started with the absorption of PP2A on DEAE-resins, followed by washing of the resins with a 0.05-0.1 M NaCl-containing buffer, which removed $\alpha_1\beta_1\delta_1$/CAB″; (4) 74-kDa δ/B″ adhered to glass surfaces, especially once it was dissociated from $\alpha_1\beta_1$/CA. This latter property may cause a loss of $\alpha_1\beta_1\delta_1$/CAB″ during the preparation. Indeed, the recovery of $\alpha_1\beta_1\delta_1$/CAB″ was improved by the addition of 0.005% Triton X-100 to the buffers throughout the purification. Triton X-100 at such a low concentration, which is less than one-third of the critical micellar concentration, is not considered to disrupt membrane structures or to disturb subcellular distributions of cellular proteins during cell fractionation (*37*).

PP2A has been reported to associate with cytoskeletal proteins such as microtubules (*38*) and neurofilaments (*39*) whose assembly and disassembly are regulated by either direct or indirect phosphorylation-dephosphorylation. The ubiquitous distribution of the 72-kDa δ/B″ in cytosol from various rat tissues, with the highest level in brain, suggests that the 72-74-kDa δ/B″ may play a role in targeting PP2A to one of the cytoskeletal proteins whose function is regulated by phosphorylation-dephosphorylation.

Previously, the 74-kDa δ/B″ in $\alpha_1\beta_1\delta_1$/CAB″ purified from human erythrocytes was shown to be phosphorylated at the serine residues by A-kinase (*7*). In this paper, the 72-kDa δ/B″ in rat brain $\alpha_1\beta_1\delta_1$/CAB″ was also phosphorylated *in vitro* by A-kinase to the same extent as human erythrocyte 74-kDa δ/B″. Recently, the *in vivo* phosphorylation of epitope-tagged B′ isoforms including B56δ was detected in 293 cells transfected with the epitope-tagged B′ constructs (*13*). A possible role of δ/B″ phosphorylation in $\alpha_1\beta_1\delta_1$/CAB″ could be the regulation of the targeting of $\alpha_1\beta_1\delta_1$/CAB″ to specific cell components, such as nuclear

and cytoskeletal proteins. On the other hand, a comparison of the molecular activities of $\alpha_1\beta_1$/CA and $\alpha_1\beta_1\delta_1$/CAB″ for various substrates suggests that δ/B″ strongly suppresses $\alpha_1\beta_1$/CA activities (*4*). The phosphorylation of δ/B″ in human erythrocyte $\alpha_1\beta_1\delta_1$/CAB″ by A-kinase slightly stimulated phosphatase activities toward phosphorylated histones and phosphorylase *a in vitro* (*7*). Therefore, another possible role of the δ/B″ phosphorylation may be to regulate the $\alpha_1\beta_1$/CA activity by changing the interaction between δ/B″ and $\alpha_1\beta_1$/CA.

We are grateful to Dr. Jean Lucus-Lenard (University of Connecticut) for her critical reading of the manuscript. We also thank Mieko Kawamura for her excellent secretarial assistance, and Ryoko Takemoto and Kenji Masuda for their skillful technical assistance.

REFERENCES

1. Cohen, P. (1989) The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58**, 453-508
2. Mumby, M.C. and Walter, G. (1993) Protein serine/threonine phosphatases: Structure, regulation, and functions in cell growth. *Physiol. Rev.* **73**, 673-699
3. Shenolikar, S. (1994) Protein serine/threonine phosphatases—new avenues for cell regulation. *Annu. Rev. Cell Biol.* **10**, 55-86
4. Usui, H., Imazu, M., Maeta, K., Tsukamoto, H., Azuma, K., and Takeda, M. (1988) Three distinct forms of type 2A protein phosphatase in human erythrocyte cytosol. *J. Biol. Chem.* **263**, 3752-3761
5. Tung, H.Y.L., Alemany, S., and Cohen, P. (1985) The protein phosphatases involved in cellular regulation: 2. Purification, subunit structure and properties of protein phosphatases-$2A_0$, $2A_1$, and $2A_2$ from rabbit skeletal muscle. *Eur. J. Biochem.* **148**, 253-263
6. Kamibayashi, C., Estes, R., Lickteig, R.L., Yang, S.-I., Craft, C., and Mumby, M.C. (1994) Comparison of heterotrimeric protein phosphatase 2A containing different B subunits. *J. Biol. Chem.* **269**, 20139-20148
7. Usui, H., Ariki, M., Miyauchi, T., and Takeda, M. (1991) Functions and properties of subunits in type 2A protein phosphatases from human erythrocyte. *Adv. Protein Phosphatases* **6**, 287-306
8. Tanabe, O., Nagase, T., Murakami, T., Nozaki, H., Usui, H., Nishito, Y., Hayashi, H., Kagamiyama, H., and Takeda, M. (1996) Molecular cloning of a 74-kDa regulatory subunit (B″ or δ) of human protein phosphatase 2A. *FEBS Lett.* **379**, 107-111
9. Csortos, C., Zolnierowicz, S., Bakó, E., Durbin, S.D., and DePaoli-Roach, A.A. (1996) High complexity in the expression of the B′ subunit of protein phosphatase $2A_0$: Evidence for the existence of at least seven novel isoforms. *J. Biol. Chem.* **271**, 2578-2588
10. Tehrani, M.A., Mumby, M.C., and Kamibayashi, C. (1996) Identification of a novel protein phosphatase 2A regulatory subunit highly expressed in muscle. *J. Biol. Chem.* **271**, 5164-5170
11. McCright, B. and Virshup, D.M. (1995) Identification of a new family of protein phosphatase 2A regulatory subunits. *J. Biol. Chem.* **270**, 26123-26128
12. Zolnierowicz, S., Van Hoof, C., Andjelković, N., Cron, P., Stevens, I., Merlevede, W., Goris, J., and Hemmings, B.A. (1996) The variable subunit associated with protein phosphatase $2A_0$ defines a novel multimember family of regulatory subunits. *Biochem. J.* **317**, 187-194
13. McCright, B., Rivers, A.M., Audlin, S., and Virshup, D.M. (1996) The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. *J. Biol. Chem.* **271**, 22081-22089
14. Resink, T.J., Hemmings, B.A., Tung, H.Y.L., and Cohen, P. (1983) Characterisation of a reconstituted Mg-ATP-dependent protein phosphatase. *Eur. J. Biochem.* **133**, 455-461
15. Foulkes, J.G. and Cohen, P. (1980) The regulation of glycogen metabolism: Purification and properties of protein phosphatase inhibitor-2 from rabbit skeletal muscle. *Eur. J. Biochem.* **105**, 195-203
16. Usui, H., Kinohara, N., Yoshikawa, K., Imazu, M., Imaoka, T., and Takeda, M. (1983) Phosphoprotein phosphatases in human erythrocyte cytosol. *J. Biol. Chem.* **258**, 10455-10463
17. Glowinski, J. and Iversen, L.L. (1966) Regional studies of catecholamines in the rat brain—I: The disposition of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]DOPA in various regions of the brain. *J. Neurochem.* **13**, 655-669
18. Koenig, H., Gaines, D., McDonald, T., Gray, R., and Scott, J. (1964) Studies of brain lysosomes—I: Subcellular distribution of five acid hydrolases, succinate dehydrogenase and gangliosides in rat brain. *J. Neurochem.* **11**, 729-743
19. Chauveau, J., Moulé, Y., and Rouiller, Ch. (1956) Isolation of pure and unaltered liver nuclei: Morphology and biochemical composition. *Exp. Cell Res.* **11**, 317-321
20. Gabriel, O. (1971) Analytical disc gel electrophoresis. *Methods Enzymol.* **22**, 565-578
21. Weber, K. and Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406-4412
22. Poehling, H.-M. and Neuhoff, V. (1981) Visualization of proteins with a silver "stain": A critical analysis. *Electrophoresis* **2**, 141-147
23. Siegel, L.M. and Monty, K.J. (1966) Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation: Application to crude preparations of sulfite and hydroxylamine reductases. *Biochim. Biophys. Acta* **112**, 346-362
24. Imaoka, T., Imazu, M., Usui, H., Kinohara, N., and Takeda, M. (1980) Isolation of an inactive component from pig heart phosphoprotein phosphatase and its reassociation with an active component. *Biochim. Biophys. Acta* **612**, 73-84
25. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
26. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
27. Dingwall, C. and Laskey, R.A. (1991) Nuclear targeting sequences—a consensus? *Trends Biochem. Sci.* **16**, 478-481
28. Wadzinski, B.E., Wheat, W.H., Jaspers, S., Peruski, L.F., Jr., Lickteig, R.L., Johnson, G.L., and Klemm, D.J. (1993) Nuclear protein phosphatase 2A dephosphorylates protein kinase A-phosphorylated CREB and regulates CREB transcriptional stimulation. *Mol. Cell. Biol.* **13**, 2822-2834
29. Jakes, S., Mellgren, R.L., and Schlender, K.K. (1986) Isolation and characterization of an inhibitor-sensitive and a polycation-stimulated protein phosphatase from rat liver nuclei. *Biochim. Biophys. Acta* **888**, 135-142
30. Turowski, P., Fernandez, A., Favre, B., Lamb, N.J.C., and Hemmings, B.A. (1995) Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression. *J. Cell Biol.* **129**, 397-410
31. Hendrix, P., Mayer-Jaekel, R.E., Cron, P., Goris, J., Hofsteenge, J., Merlevede, W., and Hemmings, B.A. (1993) Structure and expression of a 72-kDa regulatory subunit of protein phosphatase 2A: Evidence for different size forms produced by alternative splicing. *J. Biol. Chem.* **268**, 15267-15276
32. Yang, S.-D., Yu, J.-S., and Fong, Y.-L. (1986) Purification and characterization of two inactive/latent protein phosphatases from pig brain. *J. Biol. Chem.* **261**, 5590-5596
33. Patterson, C.L., Jr. and Flavin, M. (1986) A brain phosphatase with specificity for microtubule-associated protein-2. *J. Biol. Chem.* **261**, 7791-7796
34. Pitcher, J.A., Payne, E.S., Csortos, C., DePaoli-Roach, A.A., and Lefkowitz, R.J. (1995) The G-protein-coupled receptor phosphatase: A protein phosphatase type 2A with a distinct subcel-

lular distribution and substrate specificity. *Proc. Natl. Acad. Sci. USA* **92**, 8343-8347
35. Nishiwaki, S., Fujiki, H., Suganuma, M., Nishiwaki-Matsushima, R., and Sugimura, T. (1991) Rapid purification of protein phosphatase 2A from mouse brain by microcystin-affinity chromatography. *FEBS Lett.* **279**, 115-118
36. Law, B. and Rossie, S. (1995) The dimeric and catalytic subunit forms of protein phosphatase 2A from rat brain are stimulated by C_2-ceramide. *J. Biol. Chem.* **270**, 12808-12813
37. Helenius, A. and Simons, K. (1975) Solubilization of membranes by detergents. *Biochim. Biophys. Acta* **415**, 29-79
38. Sontag, E., Nunbhakdi-Craig, V., Bloom, G.S., and Mumby, M.C. (1995) A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J. Cell Biol.* **128**, 1131-1144
39. Saito, T., Shima, H., Osawa, Y., Nagao, M., Hemmings, B.A., Kishimoto, T., and Hisanaga, S. (1995) Neurofilament-associated protein phosphatase 2A: Its possible role in preserving neurofilaments in filamentous states. *Biochemistry* **34**, 7376-7384