J. Biochem. 126, 632-638 (1999)

Interconversion of Mn^{2+}-Dependent and -Independent Protein Phosphatase 2A from Human Erythrocytes: Role of Zn^{2+} and Fe^{2+} in Protein Phosphatase 2A[1]

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

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

Received June 21, 1999; accepted July 14, 1999

Human erythrocyte Mn^{2+}-dependent (C′A′) and -independent (CA) protein-serine/threonine phosphatase (PP) 2A are composed of 34-kDa catalytic C′ and C subunits, in which the metal dependency resides, and 63-kDa regulatory A′ and A subunits, respectively. Each catalytic and regulatory subunit gave the same V8- and papain-peptide maps, respectively. Stoichiometric zinc and substoichiometric iron were detected in CA but not in C′A′ [Nishito *et al.* (1999) *FEBS Lett.* 447, 29-33]. The Mn^{2+}-dependent protein-tyrosine phosphatase (PTP) activity of C′A′ was about 70-fold higher than that of CA. Pre-incubation of CA with 25 mM NaF changed CA to a Mn^{2+}-dependent form with higher PTP activity. The same NaF treatment had no effect on C′A′. Pre-incubation of C′A′ with $ZnCl_2$, zinc-metallothionein, or $FeCl_2$ activated the Mn^{2+}-independent PP activity, but pre-incubation with $FeCl_3$ did not. Ascorbate in the pre-incubation and assay mixture significantly stimulated the effect of $FeCl_2$. Pre-incubation of C′A′ with 5 μM $ZnCl_2$ and 15 μM $FeCl_2$ in the presence of 1 mM ascorbate synergistically stimulated the Mn^{2+}-independent PP activity, with concomitant suppression of the Mn^{2+}-dependent PP and PTP activities. The PP and PTP activities of CA were unaffected by the same zinc and/or iron treatment. Micromolar concentrations of vanadate strongly inhibited the Mn^{2+}-dependent PP activity of C′A′ but only slightly inhibited the PP activity of CA. Using the distinct effect of vanadate as an indicator, the interconversion between CA and C′A′ with the above mentioned treatments was proved. These results support the notion that Mn^{2+}-independent CA is a Zn^{2+}- and Fe^{2+}-metalloenzyme, whose apoenzyme is Mn^{2+}-dependent C′A′.

Key words: fluoride, protein phosphatase 2A, vanadate, zinc- and iron-metalloenzyme, zinc-metallothionein.

Protein-serine/threonine phosphatase (PP) 2A, one of the four major PPs (1, 2A, 2B, and 2C), is found in all eukaryotic cells (*1*) and plays a key role in the regulation of many cellular events, including metabolism, the cell cycle, cell proliferation, replication, transcription, translation, and viral transformation (*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 contains an additional subunit, either 51-58-kDa B (*1*), 54-74-kDa B′/B″ (*1, 3*), or 59-130-kDa PR59/PR72 (*4*).

Previously, we purified three forms of PP2A from human erythrocyte cytosol, whose subunit structures are CA ($\alpha_1\beta_1$), CAB ($\alpha_1\beta_1\gamma_1$), and CAB″ ($\alpha_1\beta_1\delta_1$), where C (α) is a 34-kDa catalytic subunit, and A (β), B (γ), and B″ (δ) are 63-, 53-, and 74-kDa regulatory subunits, respectively (*3*). Recent molecular cloning of B″ (δ) (*5*) revealed that this subunit exhibits strong sequence similarity in the central 400-amino-acid region of 54-kDa B′ (*6-8*), and is a member of the B′ family. In addition to the three Mn^{2+}-independent forms of PP2A, a Mn^{2+}-dependent form of PP2A was purified from human erythrocyte cytosol (*9*). The Mn^{2+}-dependent form is composed of a 34-kDa catalytic C′ subunit and a 63-kDa regulatory A′ subunit. The Mn^{2+} dependency resides in catalytic subunit C′ but not in regulatory subunit A′ (*9*). The V8- and papain-peptide maps of C′ and A′ were indistinguishable from those of C and A, respectively (*9*). Direct metal analysis by means of atomic absorption spectrophotometry revealed stoichiometric zinc and substoichiometric iron in CA, but no such metals in C′A′ (*9*).

In this paper, the micromolar concentration of vanadate is shown to strongly inhibit the Mn^{2+}-dependent PP activity

[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 (1992-1998).

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

Abbreviations: DSP, dual specificity phosphatase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAP, purple acid phosphatase; P-H2B histone, H2B histone phosphorylated by PKA; PKA, cAMP-dependent protein kinase; PP, protein-serine/threonine phosphatase; PTP, protein-tyrosine phosphatase; P-Tyr-Glu copolymers, Tyr-Glu copolymers phosphorylated by c-Yes; Zn-MT, zinc-metallothionein.

of C'A' but to only slightly inhibit the PP activity of CA. Using the distinct effect of vanadate as an indicator, the interconversion between CA and C'A' with the NaF treatment of CA or with the pre-incubation of C'A' with Zn^{2+} and Fe^{2+} was proved. These results further support the notion that the conventional Mn^{2+}-independent PP2A, CA, is a Zn^{2+}- and Fe^{2+}-metalloenzyme, whose apoenzyme is Mn^{2+}-dependent C'A'.

EXPERIMENTAL PROCEDURES

Materials—Calf thymus H2B histone, rabbit skeletal muscle phosphorylase *b* and phosphorylase kinase *b*, and pig heart PKA and its catalytic subunits were prepared as described previously (*3*). c-Yes was prepared from a rat liver plasma membrane fraction (*10*). [γ-^{32}P]ATP was obtained from Amersham. BSA, Tyr-Glu (1:4) copolymers (M_r = 49,100), rabbit liver zinc-metallothionein (Zn-MT) II [contains 7.2% (w/w) Zn^{2+} and 0.1% (w/w) Cd^{2+}], and sodium orthovanadate were obtained from Sigma. Ascorbate, NaF, and metal chlorides were obtained from Wako Pure Chemical Industries. Other chemicals were obtained from various commercial sources.

Preparation of Substrates—^{32}P-H2B histone and ^{32}P-phosphorylase *a* were prepared as described previously (*3*). ^{32}P-Tyr-Glu copolymers (123 nmol of bound ^{32}P/mg Tyr-Glu copolymers) were prepared as follows: A mixture (630 μl) containing 0.1 mg/ml Tyr-Glu copolymers, 20 mM Hepes-NaOH, pH 7.4, 20 mM $Mg(CH_3COO)_2$, 0.5 mM DTT, 30 μM Na_3VO_4, 0.2% (v/v) Triton X-100, 94 units/ml c-Yes, and 30 μM [γ-^{32}P]ATP ($1-2\times10^7$ cpm/nmol) was incubated for 15 h at 30°C. The reaction was stopped by adding 100% (w/v) trichloroacetic acid to a final concentration of 10%. After standing for 30 min on ice, the ^{32}P-Tyr-Glu copolymers were precipitated by centrifugation at 12,000 × *g* for 10 min at 4°C, washed twice with 0.7 ml of ice-cold 10% trichloroacetic acid and three times with 0.7 ml of ice-cold acetone, and then dried. The ^{32}P-Tyr-Glu copolymers were dissolved in 0.5 ml of 20 mM Hepes-NaOH, pH 7.4. All substrate concentrations represent the concentrations of bound ^{32}P in the substrates.

Phosphatase Assay and Protein Determination—Unless otherwise stated, PP activity was measured in a 50-μl mixture containing 50 mM Hepes-NaOH, pH 7.4, 0.5 mM DTT, 0.01% Triton X-100, 25 mM $MnCl_2$, 250 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, 0.01% Triton X-100, 7 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 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% 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 which catalyzed the release of 1 nmol of [^{32}P]P_i per minute. Protein was determined by the method of Bradford (*11*).

Purification of Mn^{2+}-Dependent (C'A') and -Independent (CA) PP2A—Purification of C'A' and CA from human erythrocyte cytosol was performed as described before (*9*). The purified C'A' and CA exhibited specific activities of 5,340-6,220 and 1,030-1,630 units/mg, respectively, under the standard assay conditions.

NaF Treatment of CA and C'A'—CA (147-235 units/ml) or C'A' (561-898 units/ml) was pre-incubated at 30°C for 10 min with or without 25 mM NaF in 10 μl of 50 mM Hepes-NaOH, pH 7.4, 0.5 mM DTT, 0.005% Triton X-100, and 10% glycerol. The NaF concentration was adjusted to 0.1 mM by dilution with the same buffer, and then phosphorylase phosphatase activity was measured at 30°C for 5 min in a 50-μl mixture containing 50 mM Hepes-NaOH, pH 7.4, 0.5 mM DTT, 0.01% Triton X-100, 10 μM ^{32}P-phosphorylase *a*, the indicated concentration of $MgCl_2$ or $MnCl_2$, and 5 μl of the diluted pre-incubation mixture. NaF at less than 0.01 and 0.05 mM had no effect on PP activity toward phosphorylase *a* and P-H2B histone, respectively. The PTP activity of 5 μl of a pre-incubation mixture whose NaF concentration was adjusted to 0.5 mM by dilution with the same buffer was measured as described under "*Phosphatase Assay*" except that 7 mM $MnCl_2$ was replaced by the indicated concentration of $MgCl_2$ or $MnCl_2$. NaF at less than 0.05 mM had no effect on the PTP activity.

Zinc and Iron Treatment of CA and C'A'—CA or C'A' (4.3-28.3 units/ml) was pre-incubated at 30°C for 15 min with the indicated concentrations of Zn-MT or $ZnCl_2$ and/or iron chlorides in a 8-48-μl mixture containing 50 mM MOPS-NaOH, pH 7.0, 0.5 mM DTT, 150 mM KCl, and 0.5 mg/ml BSA (the pre-incubation buffer), in the presence or absence of 1 mM ascorbate. The concentrations of Zn-MT represent those of the bound metal in the metalloprotein. The pre-incubated mixture was diluted if necessary as described in the legends and was assayed for PP or PTP activity. The assay for phosphorylase phosphatase activity was carried out at 30°C for 10 min in a 50-μl mixture containing 45 mM imidazole-HCl, pH 7.4, 1 mM DTT, 6 mM theophylline, 0.5 mg/ml BSA, 2 μM ^{32}P-phosphorylase *a*, and 5 μl of the pre-incubation mixture, in the presence or absence of 1 mM ascorbate. P-H2B histone phosphatase activity was measured at 30°C for 10 min in a 50-μl mixture containing 50 mM Hepes-NaOH, pH 7.4, 0.5 mM DTT, 0.01% Triton X-100, 150 mM KCl, 0.2 or 1 mM ascorbate, 100 μM ^{32}P-H2B histone, and 5 μl of the pre-incubation mixture, with or without 25 mM $MgCl_2$ or 25 mM $MnCl_2$. The PTP activity of 5 μl of the pre-incubation mixture was measured as described under "*Phosphatase Assay*" in the presence of 1 mM ascorbate.

RESULTS

Effect of NaF on the Mn^{2+} and Mg^{2+} Requirement of CA and C'A'—Since PP2A has been suggested to be a metalloenzyme and is known to be inhibited by NaF, which can react with metals in the enzyme, the effect of NaF on the Mn^{2+} and Mg^{2+} requirement of CA and C'A' was investigated (Figs. 1 and 2). CA was pre-incubated with 25 mM NaF at 30°C for 10 min and then assayed for PP activity toward phosphorylase *a*. In the assay mixture, the NaF concentration was diluted to 0.01 mM, at which NaF had no effect on the enzyme activity. The NaF treatment completely inhibited the CA activity in the absence of Mn^{2+} and Mg^{2+} (Fig. 1, upper panel). The inactivated CA could be partially reactivated by Mn^{2+} (Fig. 1, upper panel). Mg^{2+} at concentrations from 2.5 to 30 mM slightly reactivated the inactivated CA (Fig. 1, upper panel). On the other hand, the same NaF treatment of C'A' did not change the Mn^{2+}

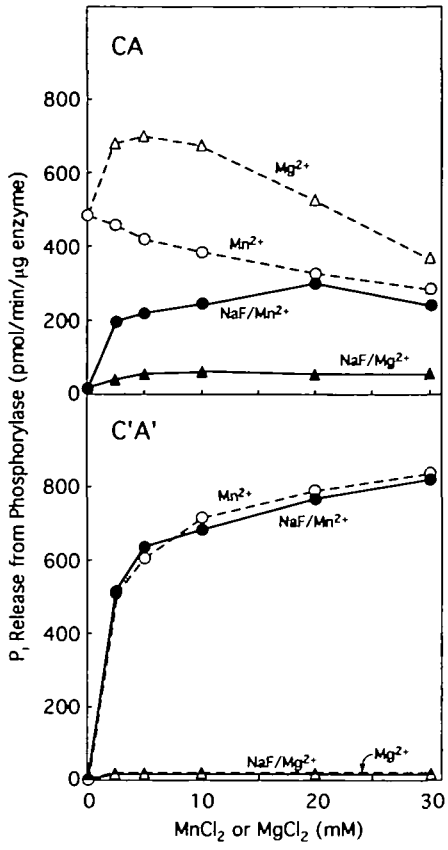

Fig. 1. **Effect of NaF treatment of CA and C'A' on the Mn^{2+} and Mg^{2+} requirement for their PP activity.** CA (147 units/ml) and C'A' (561 units/ml) were pre-incubated with (●, ▲) or without (○, △) 25 mM NaF, and then diluted 250-fold with the pre-incubation buffer. The PP activity toward phosphorylase *a* of the diluted pre-incubation mixture was measured at the indicated concentrations of $MnCl_2$ (●, ○) or $MgCl_2$ (▲, △). The NaF concentration in the reaction was adjusted to 0.01 mM. The details of the pre-incubation and the assay are given under "*NaF Treatment of CA and C'A'*" under "EXPERIMENTAL PROCEDURES."

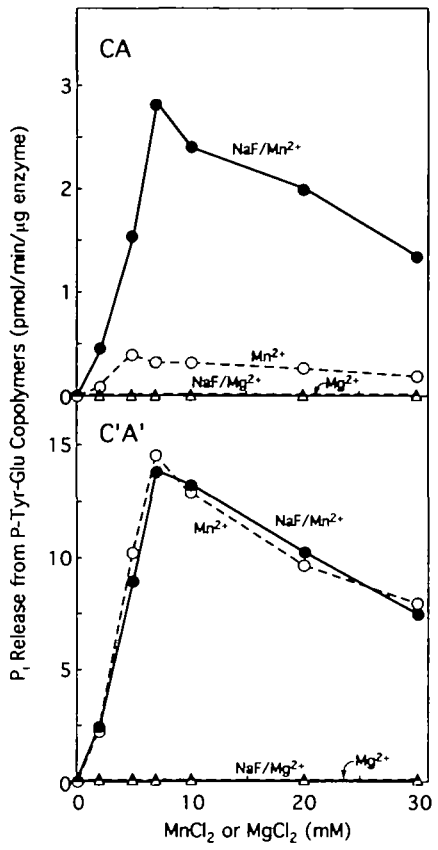

Fig. 2. **Effect of NaF treatment of CA and C'A' on the Mn^{2+} and Mg^{2+} requirement for their PTP activity.** CA (235 units/ml) and C'A' (898 units/ml) were pre-incubated with (●, ▲) or without (○, △) 25 mM NaF, and then diluted 50-fold with the pre-incubation buffer. The PTP activity of the diluted pre-incubation mixture was measured at the indicated concentrations of $MnCl_2$ (●, ○) or $MgCl_2$ (▲, △). The NaF concentration in the reaction was adjusted to 0.05 mM. The details of the pre-incubation and the assay are given under "*NaF Treatment of CA and C'A'*" under "EXPERIMENTAL PROCEDURES."

dependency (Fig. 1, lower panel). The NaF-treated CA showed similar Mg^{2+} and Mn^{2+} curves to those of C'A' rather than those of untreated CA (Fig. 1). These results indicate that the NaF treatment led to the conversion of CA to a Mn^{2+}-dependent form similar to C'A', suggesting the participation of metals in the enzyme catalysis of CA.

Effect of NaF on PTP Activity of CA and C'A'—It has been shown that PP2A has Mn^{2+}-dependent PTP activity (*12-14*). PTP activity of CA and C'A' towards P-Tyr-Glu copolymers was completely dependent on Mn^{2+}, which could not be replaced by Mg^{2+} (Fig. 2). The molecular activity of C'A' (M_r=103,000) towards P-Tyr-Glu copolymers in the presence of 7 mM Mn^{2+} was determined to be 6.21±0.32 (mean±SE, n=8) mol P_i release/min/mol C'A', *i.e.* 70-fold higher than that of CA (M_r=104,000), which was 0.090±0.003 (mean±SE, n=8) mol P_i release/min/mol CA. The K_m values of C'A' and CA for P-Tyr-Glu copolymers were 3.10±0.44 and 1.28±0.05 μM, respectively. However, the pre-incubation of CA with 25 mM NaF caused a 10-fold increase in PTP activity (Fig. 2, upper panel). The Mn^{2+} curve of NaF-treated CA was similar to that of C'A' (Fig. 2). On the other hand, the same NaF treatment of C'A' had no effect on the PTP activity (Fig. 2, lower panel). These results indicate that the NaF treatment led to the conversion of CA to a higher PTP activity form similar to C'A', suggesting the possibility that C'A' could be an apoenzyme of CA.

Effect of Zinc and Iron Pre-Incubation on PP Activity of C'A' and CA—Since Mn^{2+}-dependent C'A' did not contain zinc and iron, which were detected in CA (*9*), the effects of these metals on the Mn^{2+}-independent PP activity of C'A' and CA were investigated to determine whether or not C'A' is an apoenzyme of CA. C'A' was pre-incubated with 0.25-1,000 μM $ZnCl_2$, $FeCl_2$, or $FeCl_3$ at 30°C for 15 min in the buffer containing BSA and 150 mM KCl. An aliquot of the pre-incubation mixture was taken, diluted if necessary, and assayed for phosphorylase phosphatase activity in the absence of Mn^{2+} (Fig. 3). Pre-incubation of C'A' with 1-150 μM $ZnCl_2$ stimulated the Mn^{2+}-independent PP activity 4-5-fold (Fig. 3). Zn-MT, a ubiquitously distributed possible zinc donor for zinc-apometallo proteins (*15*), was as equally effective as $ZnCl_2$ in activating the C'A' activity (Fig. 3). On the other hand, the PP activity of C'A' was slightly stimulated by pre-incubation with 50-750 μM $FeCl_2$ (Fig. 3), but

not affected by pre-incubation with 1-500 μM $FeCl_3$ (Fig. 3). The presence of 50 μM $FeCl_2$ during the pre-incubation of C'A' with 0.5-50 μM $ZnCl_2$ rather suppressed the stimulatory effect of $ZnCl_2$ on the Mn^{2+}-independent PP activity (data not shown). Neither an additive nor a synergistic stimulatory effect of pre-incubation of C'A' with 5 μM $ZnCl_2$ and 1-1,000 μM each iron chloride was observed on the Mn^{2+}-independent PP activity (data not shown). However, the maximal Mn^{2+}-independent activity of C'A' stimulated by Zn^{2+} was about 9% of that of CA (269 pmol/min/μg enzyme). Since 1 mM ascorbate significantly enhanced the stimulatory effect of Fe^{2+} on the Mn^{2+}-independent PP activity of Mn^{2+}/Co^{2+}-dependent PP2A (*16*), the effect of ascorbate on the pre-incubation of C'A' with $ZnCl_2$ and $FeCl_2$ was investigated (Table I). The presence of 1 mM ascorbate during the pre-incubation slightly enhanced the stimulatory effect of $FeCl_2$ but not that of $ZnCl_2$ (Table I). Ascorbate at 1 mM in the assay mixture stimulated the Mn^{2+}-independent PP activity of C'A' pre-incubated with $FeCl_2$ more than 10-fold, and slightly stimulated the activity of C'A' pre-incubated with $ZnCl_2$ (Table I). When 1 mM ascorbate was present throughout the pre-incubation and assaying of C'A', the pre-incubation of C'A' with 2 μM $ZnCl_2$ and 15 μM $FeCl_2$ synergistically stimulated the Mn^{2+}-independent PP activity up to about 50% of that of CA (167 pmol/min/μg enzyme) (Table I). The synergistic stimulatory effect of $ZnCl_2$ and $FeCl_2$ on the Mn^{2+}-independent PP activity of C'A' was also observed when P-H2B histone was used as the substrate (Table II), indicating that the stimulatory effect of these metals was attributable to a direct effect on C'A' but not on the substrate. The Mn^{2+}-independent PP activity of C'A' pre-incubated with $ZnCl_2$ and $FeCl_2$ was 20% of that of CA (Table II). Such a synergistic stimulatory effect was not observed with $ZnCl_2$ or $FeCl_3$ (Table II). On the other hand, the same pre-incubation of CA with these metal chlorides had no effect on the PP activity in the presence of ascorbate (Table II). Furthermore, the ratio of PP activity of C'A' in the absence and presence of 25 mM Mg^{2+} or Mn^{2+} changed with the pre-incubation from 1.00:10.5:268 to 1.00:1.23:0.84, which is very similar to the ratio, 1.00:1.27:0.97, of CA (Table II), indicating the conversion of C'A' to CA with pre-incubation with Zn^{2+} and Fe^{2+}. Since $FeCl_3$ was partially reduced to $FeCl_2$ by ascorbate during the pre-incubation (data not shown), the slight stimulation of the Mn^{2+}-independent PP activity of C'A' on pre-incubation with $FeCl_3$ is probably due to the effect of $FeCl_2$ (Table II).

Effect of Zinc and Iron Pre-Incubation on PTP Activity of C'A'—The PTP activity of C'A' was dependent on Mn^{2+} but not on Co^{2+}, Zn^{2+}, or Fe^{2+} (data not shown). The pre-incubation of C'A' with 5 μM $ZnCl_2$ and/or 15 μM $FeCl_2$ suppressed more than 95% of the PTP activity (Table II), suggesting the conversion of C'A' to CA. The pre-incuba-

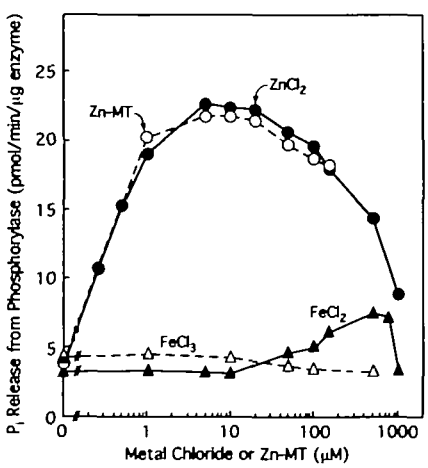

Fig. 3. **Effect of zinc and iron pre-incubation on the Mn^{2+}-independent PP activity of C'A'.** C'A' (28.3 units/ml) was pre-incubated with the indicated concentrations of Zn-MT (○), $ZnCl_2$ (●), $FeCl_2$ (▲), or $FeCl_3$ (△). The pre-incubated mixture was diluted, and 5 μl of the diluted pre-incubation mixture was used for the assaying of phosphorylase phosphatase activity in a 50-μl reaction mixture. Zn-MT, $ZnCl_2$, $FeCl_2$, and $FeCl_3$ were diluted to concentrations lower than 2.3, 3, 7.5, and 8.3 μM, respectively, at which these metals had no effect on the assay. The details of the pre-incubation and the assay are given under "*Zinc and Iron Treatment of CA and C'A'*" under "EXPERIMENTAL PROCEDURES."

TABLE I. **Effect of ascorbate on the pre-incubation of C'A' with $ZnCl_2$ and $FeCl_2$.** C'A' (28.3 units/ml) was pre-incubated with 2 μM $ZnCl_2$ and/or 15 μM $FeCl_2$ in the presence or absence of 1 mM ascorbate. After 3-fold dilution with the pre-incubation buffer, 5 μl of the pre-incubation mixture was used for the assaying of Mn^{2+}-independent phosphorylase phosphatase activity in the presence or absence of 1 mM ascorbate in a 50-μl reaction mixture. $ZnCl_2$ and $FeCl_2$ in the diluted pre-incubation mixture had no effect on the assay for PP activity. The details are given under "*Zinc and Iron Treatment of CA and C'A'*" under "EXPERIMENTAL PROCEDURES."

Pre-incubated with	Ascorbate	PP activity (pmol P_i/min/μg enzyme) in the presence of	
		None	Ascorbate
None	−	6.3	11.3
$ZnCl_2$	−	22.2	30.5
$FeCl_2$	−	6.9	70.7
$ZnCl_2+FeCl_2$	−	14.3	78.9
None	+	6.0	9.4
$ZnCl_2$	+	20.3	27.4
$FeCl_2$	+	12.1	49.9
$ZnCl_2+FeCl_2$	+	24.0	83.2

TABLE II. **Effect of zinc and iron pre-incubation on the Mg^{2+} and Mn^{2+} requirement for PP and PTP activities of C'A' and CA.** C'A' and CA (4.3 units/ml each) were pre-incubated with 5 μM zinc and/or 15 μM iron chlorides, and then their PP activities toward P-H2B histone in the presence or absence of 25 mM divalent cations and PTP activity were measured, as described under "*Zinc and Iron Treatment of CA and C'A'*" under "EXPERIMENTAL PROCEDURES." Ascorbate, at 1 mM, was present throughout the experiments.

Phosphatase	Pre-incubated with	PP activity (pmol P_i/min/μg enzyme) in the presence of			PTP activity (fmol P_i/min/μg enzyme)
		None	Mg^{2+}	Mn^{2+}	
C'A'	None	25	263	6,690	2,140
C'A'	$ZnCl_2$	128	190	168	38.0
C'A'	$FeCl_2$	220	458	650	72.8
C'A'	$FeCl_3$	65	325	1,430	322
C'A'	$ZnCl_2+FeCl_2$	558	685	470	36.5
C'A'	$ZnCl_2+FeCl_3$	140	218	193	41.0
CA	None	2,740	3,480	2,660	12.9
CA	$ZnCl_2$	2,860	3,620	2,470	11.4
CA	$FeCl_2$	2,970	3,620	2,600	10.6
CA	$FeCl_3$	2,980	3,590	2,550	12.1
CA	$ZnCl_2+FeCl_2$	2,780	3,610	2,540	9.8
CA	$ZnCl_2+FeCl_3$	2,990	3,740	2,480	9.8

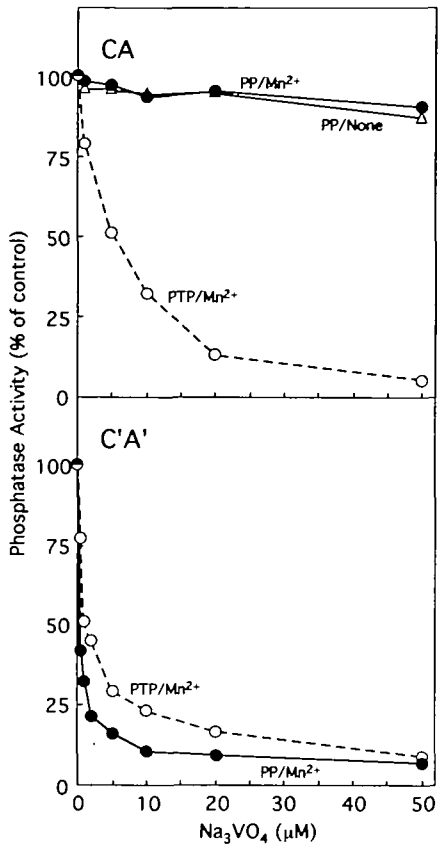

Fig. 4. **Effect of vanadate on the PP and PTP activities of CA and C′A′.** The PP activity toward P-H2B histone in the presence (●) or absence (△) of 25 mM $MnCl_2$ was measured under the standard assay conditions except that 250 mM NaCl was replaced by 150 mM KCl, and the indicated concentration of Na_3VO_4 was included. The PTP activity (○) was measured as described under "*Phosphatase Assay and Protein Determination*" under "EXPERIMENTAL PROCEDURES" except that the indicated concentration of Na_3VO_4 was included. CA (0.34 unit/ml, upper panel) or C′A′ (0.43 unit/ml, lower panel) was used.

tion of C′A′ with 15 μM $FeCl_3$ also suppressed the PTP activity, but $FeCl_3$ was less effective than $FeCl_2$ (Table II). However, the PTP activity of CA was only slightly suppressed by pre-incubation with these metal chlorides (Table II). These results also suggest that Zn^{2+} and Fe^{2+} in CA suppress PTP activity.

Effect of Vanadate on PP Activity of CA, C′A′, and Their Modified Forms—It has been shown that vanadate at micromolar concentrations strongly inhibits PTPs and DSPs but not PPs (*17*). The Mn^{2+}-dependent PTP activity of CA was inhibited by vanadate with an IC_{50} value of 5.3 μM, but the PP activity of CA in the presence or absence of 25 mM $MnCl_2$ was only slightly inhibited by 1-50 μM vanadate (Fig. 4, upper panel). On the other hand, the Mn^{2+}-dependent PP and PTP activities of C′A′ were strongly inhibited by vanadate with IC_{50} values of 0.4 and 1.2 μM, respectively (Fig. 4, lower panel). Using the distinct effect of vanadate on the PP activity of CA and C′A′ as an indicator, the possible interconversion between CA and C′A′ with the above mentioned treatments was proved (Table III). NaF treatment changed CA to a Mn^{2+}-dependent form similar to C′A′ (Figs. 1 and 2). The Mn^{2+}-dependent PP activity of the NaF-treated CA was strongly inhibited by 5 μM vanadate (Table III). On the other hand, pre-incubation of C′A′ with 5 μM $ZnCl_2$ and 15 μM $FeCl_2$ in the presence of 1 mM ascorbate changed C′A′ to a Mn^{2+}-independent form similar to CA (Table II). The PP activity in the presence of 25 mM $MnCl_2$ of the Zn^{2+}- and Fe^{2+}-treated C′A′ was rather resistant to 5 μM vanadate (Table III). These results further support the theory that CA and C′A′ are interconvertible, and that C′A′ is an apoenzyme of CA, a Zn^{2+}- and Fe^{2+}-metalloenzyme.

TABLE III. **Effect of vanadate on PP activity of NaF-treated CA, and Zn^{2+}- and Fe^{2+}-pre-incubated C′A′.** Treatment of CA (235 units/ml) with 25 mM NaF, and pre-incubation of C′A′ (5.9 units/ml) with 5 μM $ZnCl_2$ and 15 μM $FeCl_2$ were carried out as described under "EXPERIMENTAL PROCEDURES." After 50-fold dilution of the treated CA mixture, the PP activity of the treated CA was measured at 30°C for 10 min in a 50-μl mixture containing 50 mM Hepes-NaOH, pH 7.4, 0.5 mM DTT, 0.01% Triton X-100, 150 mM KCl, 25 mM $MnCl_2$, 100 μM ^{32}P-H2B histone, and 5 μl of the treated enzyme solution, with or without 5 μM Na_3VO_4. The assay mixture for the treated C′A′ contained 0.2 mM ascorbate.

Phosphatase	Treated with	Vanadate	PP activity (pmol P_i/min/μg enzyme)	Effect of vanadate (%)
CA	None	−	2,140	100
		+	2,110	99
CA	NaF	−	1,940	100
		+	526	27
C′A′	None	−	5,040	100
		+	1,290	26
C′A′	$ZnCl_2$+$FeCl_2$	−	418	100
		+	364	87

DISCUSSION

Previously, a Mn^{2+}-dependent form (C′A′) and a Mn^{2+}-independent conventional form (CA) of PP2A were purified from human erythrocyte cytosol to electrophoretic homogeneity (*9, 18*). Cross hybridization of the catalytic (C and C′) and regulatory (A and A′) subunits of Mn^{2+}-dependent C′A′ and Mn^{2+}-independent CA revealed that the Mn^{2+} dependency resides in catalytic subunit C′ but not in regulatory subunit A′ (*9*). The Mn^{2+}-dependent catalytic C′ subunit was suggested to be an apoenzyme of the Mn^{2+}-independent catalytic C subunit for the following reasons (*9*); (i) C and C′ comigrated on SDS-PAGE; (ii) the V8- and papain-peptide maps of C′ were indistinguishable from those of C; (iii) C and C′ could cross hybridize with A and A′ to form CA′ and C′A; (iv) stoichiometric zinc and substoichiometric iron were detected in CA but not in C′A′; (v) when 1 mM EDTA was added to the buffers for C′A′ purification, the C′A′/CA ratio in the DEAE-Toyopearl elution profile (*9*) changed significantly from 8.1:100 to 13.2:100; and (vi) the pre-incubation with Zn^{2+} and Fe^{2+} in the presence of 1 mM ascorbate irreversibly changed C′ to a Mn^{2+}-independent form similar to C, but had no effect on C. In this study, this notion was further confirmed by the following evidence; (i) incubation of CA with NaF, which can react with metals in the enzyme, changed CA to a Mn^{2+}-dependent form similar to C′A′, but NaF had no effect on C′A′ (Figs. 1 and 2); (ii) the pre-incubation of C′A′ with Zn^{2+} and Fe^{2+} in the presence of 1 mM ascorbate increased the Mn^{2+}-independent activity up to 20–50% of that of CA, with concomitant decreases in the Mn^{2+}-de-

pendent PP and PTP activities (Tables I and II); and (iii) the interconversion between C'A' and CA was proved by the differential effects of vanadate on the PP activities of C'A' and CA (Table III).

Pre-incubation of C'A' with Fe^{2+} in the presence of ascorbate significantly stimulated the Mn^{2+}-independent PP activity (Tables I and II). Since Fe^{2+} was stabilized in its +2 state by DTT during the pre-incubation with C'A' (data not shown), ascorbate, as a reducing agent, may not only stabilize the valency state of Fe^{2+}, but also may allow Fe^{2+} to fit properly on C'. In fact, the presence of ascorbate during the pre-incubation of C'A' was essential to obtain the synergistic stimulatory effect of Fe^{2+} and Zn^{2+} on the Mn^{2+}-independent PP activity (Table I).

The crystal structures of the catalytic subunits of PP1 (*19, 20*) and PP2B (*21, 22*) revealed a common motif containing a dinuclear metal ion center located at the active site. Every active site residue of PP1 and PP2B involved in metal coordination or implicated in catalysis is strictly conserved in PP2A. The catalytic subunits of PP1 have also been shown to exist in two forms: an active form and an inactive or latent form which requires Co^{2+} or Mn^{2+} for activity (*23, 24*). The bacterially expressed catalytic subunit of PP1 requires exogenous Mn^{2+} or Co^{2+} for its activity, whereas the enzyme freshly purified from mammalian tissues does not (*25, 26*). The Mn^{2+}- or Co^{2+}-dependent recombinant PP1 was also activated by a combination of Fe^{2+}/Zn^{2+}, but not by the individual metals (*27*). PP2B has been shown to be protected by superoxide dismutase and ascorbate, suggesting that Fe^{2+}-Zn^{2+}-PP2B is the active form of the enzyme (*28*). Yu (*16*) obtained Mn^{2+}- and Co^{2+}-dependent PP2A after prolonged storage of spontaneously active PP2A and observed the activation of the latent form of PP2A on the addition of Fe^{2+} and reducing agents such as ascorbate or DTT to the reaction mixture, suggesting that Fe^{2+} could be a biologically important cofactor responsible for PP2A activation. The crystal structures of PP1 and PP2B (*19-22*) also showed the presence of a dinuclear metal center with a coordination environment identical to the PAP active site, except for the absence of the tyrosinate ligated to Fe^{3+} that gives the PAPs their characteristic purple color. Although the active state of the mammalian PAPs is clearly the Fe^{3+} Fe^{2+} oxidation state (*29*), the metal content and active oxidation state of the PPs are still the subject of some debate. In this study, C'A' of human erythrocyte PP2A was activated by Zn^{2+} and/or Fe^{2+}. The specific activity of Fe^{2+}- or Zn^{2+}-activated C'A' was about 20-50% of that of CA, suggesting that Fe^{2+}-Zn^{2+}-PP2A is the active form of the enzyme.

The irreversible activation of C'A' on pre-incubation with Zn-MT (Fig. 3) is consistent with the notion that C'A' is an apoenzyme of CA in which stoichiometric zinc was detected (*9*). It has been shown that rat hepatic Zn-MT successfully reactivated apoenzymes prepared from yeast aldolase, thermolysin, *Escherichia coli* alkaline phosphatase, and bovine erythrocyte carbonic anhydrase (*15*). This activation was as good as, or better than, that obtained with simple zinc salts. Reversible zinc exchange between Zn-MT and the estrogen receptor, a zinc finger-containing transcription factor, has also been observed *in vitro* (*30*).

PP2A has been shown to possess Mn^{2+}-dependent PTP activity, although the activity is less than one percent of that of authentic PTPs (*12-14*). A protein factor which stimulates the PTP activity of PP2A has been reported (*14*), but the physiological importance of the PTP activity remains controversial. The Mn^{2+}-dependent PTP activity of C'A' was much higher than that of CA, and was suppressed by pre-incubation of C'A' with $ZnCl_2$ and $FeCl_2$ (Table II). On the other hand, the PTP activity of CA increased significantly on pre-incubation of CA with NaF, which can react with metals in the enzyme (Fig. 2). These results suggest that zinc and/or iron in CA may suppress the intrinsic Mn^{2+}-dependent PTP activity. Unlike PP1, PP2A, and PP2B, PTP is insensitive to NaF, is inhibited by micromolar concentrations of zinc (*31*) or vanadate (*32, 33*), does not require metal ions but uses a cysteine nucleophile for catalysis, and forms a phosphoenzyme intermediate (*34*). The Mn^{2+}-dependent PP and PTP activities of C'A' were also strongly inhibited by micromolar concentrations of zinc (Table II) and vanadate (Fig. 4), while the PP activity of CA was not significantly inhibited by 1-50 μM vanadate (Fig. 4). These results suggest that the Mn^{2+}-dependent PP and PTP activities of C'A' may be catalyzed by a mechanism, possibly through a phosphoenzyme intermediate, different from the mechanism of CA catalysis, which may proceed through the direct attack of an metal-activated water molecule at the phosphorus center of the substrate, without phosphoryl transfer to the enzyme (*20*). The physiological significance of the Mn^{2+}-dependent form of PP2A remains to be explored.

We are grateful to Dr. Jean Lucas-Lenard (University of Connecticut) for critical reading of the manuscript. We also thank Dr. Masahiro Ariki for preparing c-Yes, and Mieko Kawamura and Ryoko Takemoto for their excellent secretarial and 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. 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
4. 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
5. Tanabe, O., Gomez, G.A., Nishito, Y., Usui, H., and Takeda, M. (1997) Molecular heterogeneity of the cDNA encoding a 74-kDa regulatory subunit (B″ or δ) of human protein phosphatase 2A. *FEBS Lett.* **408**, 52-56
6. McCright, B. and Virshup, D.M. (1995) Identification of a new family of protein phosphatase 2A regulatory subunits. *J. Biol. Chem.* **270**, 26123-26128
7. 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
8. 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
9. Nishito, Y., Usui, H., Shinzawa-Itoh, K., Inoue, R., Tanabe, O., Nagase, T., Murakami, T., and Takeda, M. (1999) Direct metal

analyses of Mn^{2+}-dependent and -independent protein phosphatase 2A from human erythrocytes detect zinc and iron only in the Mn^{2+}-independent one. *FEBS Lett.* **447**, 29-33
10. Ariki, M., Tanabe, O., Usui, H., Hayashi, H., Inoue, R., Nishito, Y., Kagamiyama, H., and Takeda, M. (1997) Identification of autophosphorylation sites in c-Yes purified from rat liver plasma membranes. *J. Biochem.* **121**, 104-111
11. 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
12. Chernoff, J., Li, H.-C., Cheng, Y.-S.E., and Chen, L.B. (1983) Characterization of a phosphotyrosyl protein phosphatase activity associated with a phosphoseryl protein phosphatase of M_r = 95,000 from bovine heart. *J. Biol. Chem.* **258**, 7852-7857
13. Foulkes, J.G., Erikson, E., and Erikson, R.L. (1983) Separation of multiple phosphotyrosyl- and phosphoseryl-protein phosphatases from chicken brain. *J. Biol. Chem.* **258**, 431-438
14. Cayla, X., Van Hoof, C., Bosch, M., Waelkens, E., Vandekerckhove, J., Peeters, B., Merlevede, W., and Goris, J. (1994) Molecular cloning, expression, and characterization of PTPA, a protein that activates the tyrosyl phosphatase activity of protein phosphatase 2A. *J. Biol. Chem.* **269**, 15668-15675
15. Brady, F.O. (1982) The physiological function of metallothionein. *Trends Biochem. Sci.* **7**, 143-145
16. Yu, J.-S. (1998) Activation of protein phosphatase 2A by the Fe^{2+}/ascorbate system. *J. Biochem.* **124**, 225-230
17. Hunter, T. (1995) Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* **80**, 225-236
18. 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
19. Goldberg, J., Huang, H.-B., Kwon, Y.-G., Greengard, P., Nairn, A.C., and Kuriyan, J. (1995) Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* **376**, 745-753
20. Egloff, M.-P., Cohen, P.T.W., Reinemer, P., and Barford, D. (1995) Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. *J. Mol. Biol.* **254**, 942-959
21. Griffith, J.P., Kim, J.L., Kim, E.E., Sintchak, M.D., Thomson, J.A., Fitzgibbon, M.J., Fleming, M.A., Caron, P.R., Hsiao, K., and Navia, M.A. (1995) X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell* **82**, 507-522
22. Kissinger, C.R., Parge, H.E., Knighton, D.R., Lewis, C.T., Pelletier, L.A., Tempczyk, A., Kalish, V.J., Tucker, K.D., Showalter, R.E., Moomaw, E.W., Gastinel, L.N., Habuka, N., Chen, X., Maldonado, F., Barker, J.E., Bacquet, R., and Villafranca, J.E. (1995) Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature* **378**, 641-644
23. Villa-Moruzzi, E., Ballou, L.M., and Fischer, E.H. (1984) Phosphorylase phosphatase: Interconversion of active and inactive forms. *J. Biol. Chem.* **259**, 5857-5863
24. Chu, Y., Wilson, S.E., and Schlender, K.K. (1994) A latent form of protein phosphatase 1α associated with bovine heart myofibrils. *Biochim. Biophys. Acta* **1208**, 45-54
25. Zhang, Z., Bai, G., Deans-Zirattu, S., Browner, M.F., and Lee, E.Y.C. (1992) Expression of the catalytic subunit of phosphorylase phosphatase (protein phosphatase-1) in *Escherichia coli*. *J. Biol. Chem.* **267**, 1484-1490
26. Alessi, D.R., Street, A.J., Cohen, P., and Cohen, P.T.W. (1993) Inhibitor-2 functions like a chaperone to fold three expressed isoforms of mammalian protein phosphatase-1 into a conformation with the specificity and regulatory properties of the native enzyme. *Eur. J. Biochem.* **213**, 1055-1066
27. Chu, Y., Lee, E.Y.C., and Schlender, K.K. (1996) Activation of protein phosphatase 1: Formation of a metalloenzyme. *J. Biol. Chem.* **271**, 2574-2577
28. Wang, X., Culotta, V.C., and Klee, C.B. (1996) Superoxide dismutase protects calcineurin from inactivation. *Nature* **383**, 434-437
29. Merkx, M. and Averill, B.A. (1998) Ga^{3+} as a functional substitute for Fe^{3+}: Preparation and characterization of the Ga^{3+} Fe^{2+} and Ga^{3+} Zn^{2+} forms of bovine spleen purple acid phosphatase. *Biochemistry* **37**, 8490-8497
30. Cano-Gauci, D.F. and Sarkar, B. (1996) Reversible zinc exchange between metallothionein and the estrogen receptor zinc finger. *FEBS Lett.* **386**, 1-4
31. Brautigan, D.L., Bornstein, P., and Gallis, B. (1981) Phosphotyrosyl-protein phosphatase: Specific inhibition by Zn^{2+}. *J. Biol. Chem.* **256**, 6519-6522
32. Leis, J.F. and Kaplan, N.O. (1982) An acid phosphatase in the plasma membranes of human astrocytoma showing marked specificity toward phosphotyrosine protein. *Proc. Natl. Acad. Sci. USA* **79**, 6507-6511
33. Swarup, G., Speeg, Jr., K.V., Cohen, S., and Garbers, D.L. (1982) Phosphotyrosyl-protein phosphatase of TCRC-2 cells. *J. Biol. Chem.* **257**, 7298-7301
34. Denu, J.M., Stuckey, J.A., Saper, M.A., and Dixon, J.E. (1996) Form and function in protein dephosphorylation. *Cell* **87**, 361-364