An inactive protein phosphatase 2A population is associated with methylesterase and can be re-activated by the phosphotyrosyl phosphatase activator

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We have described recently the purification and cloning of PP2A (protein phosphatase 2A) leucine carboxylmethyltransferase. We studied the purification of a PP2A-specific methylesterase that co-purifies with PP2A and found that it is tightly associated with an inactive dimeric or trimeric form of PP2A. These inactive enzyme forms could be reactivated as Ser/Thr phosphatase by PTPA (phosphotyrosyl phosphatase activator of PP2A). PTPA was described previously by our group as a protein that stimulates the *in vitro* phosphotyrosyl phosphatase activity of PP2A; however, PP2A-specific methyltransferase could not bring about the

activation. The PTPA activation could be distinguished from the Mn²⁺ stimulation observed with some inactive forms of PP2A, also found associated with PME-1 (phosphatase methyl-esterase 1). We discuss a potential new function for PME-1 as an enzyme that stabilizes an inactivated pool of PP2A.

Key words: methylation, methylesterase, phosphorylation, phosphotyrosyl phosphatase activator, protein phosphatase 2A (PP2A), signal transduction.

INTRODUCTION

PP2A (protein phosphatase 2A) is one of the major Ser/Thr phosphatases known to be implicated in the regulation of many cellular processes [1]. It is believed that the complex composition and regulation of PP2A provide the molecular basis for the appropriate regulation of these numerous cellular processes. The core structure is built from a 36 kDa catalytic subunit (PP2A_C) and a constant 65 kDa regulatory subunit (PR65 or A subunit). This core structure can exist as such [2] or it can be associated with a third variable subunit. Three major classes of the third subunit have been described so far: PR55/B, PR61/B' and PR72/B''. Each of the subunits of PP2A exists in at least two isoforms, leading to a multitude of assembly combinations of holoenzymes, partially explaining the multiple and diverse cellular functions of PP2A. Although no obvious sequence similarity exists among these third-subunit families, two A-subunit-binding domain motifs have been found to be present in all of them [3,4]. Furthermore, PP2A can be associated with and regulated by a still growing number of other cellular and viral proteins [1].

The kinase–phosphatase relationship appears to be more intimate than anticipated previously on the basis of the general antagonism between both classes of enzymes. Indeed, signal-transduction ‘cassettes’ have been discovered wherein a protein kinase and PP2A interacted directly (see [5] for an overview). In other cases, the kinase and PP2A are linked via a common anchoring protein [6,7] or they are present in the same complex via their own partners [8–11]. In future, an understanding of these kinase–phosphatase couples and how they are regulated will be of utmost importance to have a deeper insight of the signalling pathways wherein they are involved.

The fact that PP2A is found in so many different complexes led to the concept that the structure–function relationship of PP2A is not static but may be highly dynamic and flexible, being equipped with the possibility to react to a plethora of signals.

In sharp contrast with this view, the biochemical basis for such a dynamic regulation is still missing, although post-translational modifications have been described. Phosphorylation at Tyr³⁰⁷ by several tyrosine-specific kinases [12–14] or at an unidentified threonine residue of PP2A_C by an autoactivated kinase [15,16] was found to inactivate the phosphatase activity. Most of the PR61 proteins (except PR61γ) were found to be phosphoproteins *in vivo* [17], and phosphorylation of PR61δ *in vitro* by the cAMP-dependent protein kinase was shown to change the substrate specificity of the trimeric holoenzyme, without dissociation of the PR61 subunit [18]. Ca²⁺ has been shown to regulate PP2A_{T72} directly, probably by a conformational change of the two EF-hand motifs in the PR72 subunit [19]. (To indicate a particular composition of PP2A, a suffix is used: D for dimeric and T for trimeric, followed by the indication of the third variable subunit. For example, PP2A_{iT55} is the inactive trimeric enzyme form with PR55 as the third variable subunit.) In addition to these post-translational modifications, a unique type of reversible methylation occurs at the carboxy group of the C-terminal Leu³⁰⁹ of PP2A_C [20–24]. Methylation of Leu³⁰⁹ is catalysed by a specific, 38 kDa, AdoMet (S-adenosyl-methionine)-dependent methyltransferase, namely LCMT (leucine carboxylmethyltransferase), which was purified, cloned and expressed as an active recombinant protein in bacteria [25]. This methylation is reversible and demethylation is catalysed by a specific protein PME-1 (phosphatase methylesterase 1), which was purified by following its activity [26] and subsequently cloned

Abbreviations used: AdoMet, S-adenosylmethionine; DTT, dithiothreitol; EST, expressed sequence tag; LCMT, leucine carboxylmethyltransferase; PME-1, phosphatase methylesterase 1; pNPP, p-nitrophenyl phosphate; PP2A, protein phosphatase 2A; PP2A_i, inactive form of PP2A; PTPA, phosphotyrosyl phosphatase activator of PP2A (or phosphatase two A phosphatase activator).

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as a protein that was stably associated with two inactive mutants of PP2A [27].

The C-terminal sequence of PP2A_C is well conserved over different phyla, and several amino acids at the C-terminus have been shown to be important in the binding of the third subunits to the core structure of PP2A [28–30]. Also, methylation of PP2A_C was suggested to be a modulating factor in third-subunit binding in yeast [30–32] as well as in mammals [33–36]. Two inactive synthetic mutants of PP2A were shown to interact with PME-1, whereas active PP2A did not bind [27]. The significance of this observation could have been limited to the fact that it was instrumental for the isolation and cloning of PME-1. In the present study, we report the existence of some probable physiologically inactive forms of PP2A. We started the purification of methylesterase from porcine brain and rabbit skeletal muscles and found that, in addition to 'free' methylesterase activity, a major activity was found in a complex with inactive PP2A that could be recovered by incubation with PTPA (phosphotyrosyl phosphatase activator of PP2A). Previously, PTPA was characterized, purified and cloned by our group as an *in vitro* activator of the intrinsic tyrosyl phosphatase activity of PP2A [37–39]. Therefore PTPA can also stimulate the Ser/Thr phosphatase activity of this inactive pool of PP2A complexed with PME-1. After activation, PP2A dissociated from PME-1 and it could no longer be distinguished from active PP2A as purified from tissues. We mentioned the existence of such an inactive form of PP2A as unpublished, non-documented results in [1].

MATERIALS AND METHODS

Proteins, antibodies, materials and assays

Recombinant LCMT [25] and PTPA [38] were purified as described previously. The dimeric (PP2A_D) and trimeric (PP2A_{T55} and PP2A_{T72}) holoenzyme forms of PP2A, with PR55/B and PR72/B'' respectively as the third variable subunit, were purified from rabbit skeletal muscles [40]. Ser/Thr phosphatase activity of PP2A was measured using ³²P-labelled phosphorylase *a* as the substrate in the presence and absence of 33 µg/ml protamine and 16 mM ammonium sulphate as described previously [41]. Inactive PP2A was measured under the same conditions after a 10 min preincubation by an activation step with a saturating amount of pure PTPA, 5 mM MgCl₂ and 1 mM ATP at 30 °C. For calculating the inactive units, the units measured without PTPA/ATP-Mg²⁺ were subtracted. For calculating the specific activity, proteins were assessed by a densitometric scan of a Coomassie Blue-stained polyacrylamide gel in comparison with a standard curve of BSA. Tyrosyl phosphatase activity of PP2A was measured using either [³²P]phosphotyrosyl RCAM (reduced carboxamide methylated and maleylated lysozyme) or pNPP (*p*-nitrophenyl phosphate) as the substrate after 10 min activation with a saturating amount of PTPA in the presence of 5 mM MgCl₂ and 1 mM ATP as described previously [39,42]. Alternatively, pNPP was used as the substrate as described in [42], but with adapted volumes to be used in a miniature version of a 96-well plate reader. LCMT was measured as described in [25]. Immunodetection by Western-blot analysis was performed as described in [43] with the following: mouse monoclonal antibodies to PP2A_C (clone F2. 6A10) and PR65 (clone C5.3D10) (gifts from S. Dilworth, Hammersmith Hospital, Imperial College, London, U.K.), rabbit polyclonal anti-recombinant PR55 (no. 22, used at 1:2000; a gift from B. Hemmings, FMI, Basel, Switzerland), a rabbit methylation-sensitive anti-peptide antiserum raised against residues 299–309 of PP2A_C (used at 1:2000) [25] or rabbit

polyclonal anti-recombinant PME-1 (used at 1:2000; the present study).

PP2A methylesterase assay

A semi-quantitative assay was based on the methylester turnover as demonstrated previously [26]. It is composed of a three-component system (PP2A, LCMT, PME-1) and AdoMet for the reversible methylation–demethylation of the PP2A C-terminus, wherein multiple rounds of methylation and demethylation improve the sensitivity substantially. As the source of methyltransferase that specifically methylates Leu³⁰⁹ of PP2A_C, we used 5–10 nM bacterially expressed recombinant LCMT, which was produced and purified by the method of de Baere et al. [25], 2.5 µM [³H]AdoMet (5000–10 000 c.p.m./pmol), 2 nM PP2A_D in 20 mM Tris/HCl (pH 7.4) and 3 mM DTT (dithiothreitol) in 5 µl and we added 5 µl of a source of methylesterase. This mixture was incubated at 30 °C in an open glass vial (40 mm × 8 mm), placed in a sealed scintillation vial containing 5 ml of scintillation fluid. The volatile [³H]methanol produced by the methylesterase reaction diffused into the scintillation fluid and was measured after 60 and 120 min when the amount of [³H]AdoMet was not yet a limiting factor. The amount of [³H]methanol produced was a function of the methylesterase concentration. However, most of the tissue methylesterase contained also an unpredictable amount of endogenous source of PP2A, which was also the target for a methylation–demethylation reaction and, therefore, influenced the absolute production of methanol. Therefore this assay is only semi-quantitative, but has the advantage of being very sensitive and does not require the preparation of a ³H-methylated PP2A before the assay. Control experiments with omission of LCMT and/or PP2A assured that methanol production was dependent on methylation–demethylation of PP2A.

Purification of methylesterase from porcine brain

Fresh porcine brains (1 kg), collected in a local slaughterhouse, were homogenized in 1 litre of buffer A [50 mM Tris/HCl, pH 8.0/0.5 mM DTT/1 mM EDTA/1 mM EGTA/0.1 mM PMSF/0.1 mM tosyl-l-lysylchloromethane ('TLCK')/0.5 mM benzamide/250 mM sucrose]. The homogenate was centrifuged for 20 min at 10 000 *g* and the supernatant adsorbed twice (batchwise) on 800 ml of DEAE-Sephacel, equilibrated in buffer B (20 mM Tris/HCl, pH 7.4/0.5 mM DTT/1 mM EDTA/1 mM EGTA/0.5 mM benzamide) in a funnel with a sintered glass filter. DEAE-Sephacel was washed five times with 200 ml of buffer B and was eluted nine times with 200 ml of buffer B containing 0.15 M NaCl and then eluted twice with 500 ml of buffer B containing 0.4 M NaCl to remove most of the PP2A and residual methylesterase. Eluates 3–9 of 0.15 M NaCl and 0.4 M NaCl were both processed as follows: after pooling, solid ammonium sulphate was added to a 60% saturation. After 30 min, the mixture was centrifuged for 30 min at 10 000 *g*; the pellet was resuspended in buffer B (approx. 80 ml) and stored at –20 °C after dialysis for 1 h against buffer B. In consecutive purifications, the 0.15 M NaCl or the 0.4 M NaCl fraction was processed further without freezing and storage, alternating the procedure for both fractions. The volume was adapted to obtain the same conductivity as 0.8 M ammonium sulphate in buffer B (total volume, approx. 120 ml) and for the stored frozen fraction, the adaptation to 0.8 M ammonium sulphate was made after thawing. No differences were observed in using one or the other sequence in the procedure. The fraction in 0.8 M ammonium sulphate was loaded on to a phenyl-Sepharose column (2.5 cm × 15 cm,

equilibrated in buffer B containing 0.8 M ammonium sulphate) and the column was washed with 70 ml of the equilibration buffer. The column was developed with a descending gradient of 0.8–0 M ammonium sulphate and a concurrent gradient of 0–20 % (v/v) glycerol in 600 ml of buffer B. During the purification of methylesterase from the 0.15 M NaCl fraction, LCMT (see Figure 1 in [41]) and PP1 (results not shown) eluted during the descending gradient and 'free' methylesterase eluted near the end of the gradient. Most of the methylesterase (estimated at > 90 % of the total activity) could be eluted from the column using 150 ml of 20 % glycerol followed by 150 ml of 50 % (v/v) ethylene glycol, co-eluting with PP2A activity. The active fractions were pooled and loaded on to a DEAE-Sephacel column (2.5 cm × 15 cm), equilibrated in buffer B; after washing with 2 bed vol. of buffer B, the column was developed with a 600 ml gradient of 0–0.6 M NaCl in buffer B. Methylesterase and PP2A activities co-eluted as a rather broad peak at approx. 0.35 M NaCl. The active fractions were pooled and concentrated by 60 % ammonium sulphate precipitation. The pellet was resuspended in 10 ml of buffer B and submitted to ACA34 gel filtration (2.5 cm × 95 cm). The fractions containing methylesterase were pooled (approx. 50 ml) and loaded on to a polylysine-Sephacel column (1.5 cm × 12 cm), equilibrated in buffer B. After washing with 2 bed vol. of buffer B, the column was developed with a 200 ml gradient of 0–0.6 M NaCl in buffer B; methylesterase eluted at approx. 0.35 M NaCl and PP2A activity eluted at approx. 0.47 M NaCl. The active methylesterase fractions were pooled and concentrated by consecutive dialyses against 50 % (w/v) poly(ethylene glycol) and 60 % glycerol in buffer B to a volume of approx. 2 ml. This fraction was finally loaded on to a Mono Q 5/5 HR or Resource Q column (Amersham Biosciences) equilibrated in buffer B. After washing the column with 5 ml of 0.2 M NaCl in the same buffer, the column was developed with 25 ml of a 0.2–0.5 M NaCl gradient in buffer B. PP2A (eluting at approx. 0.3 M NaCl) was almost completely separated from the methylesterase activity (eluting at approx. 0.35 M NaCl; see the Results section). The active fractions of methylesterase were pooled, concentrated by dialysis against 60 % glycerol in buffer B and stored at –20 °C.

Protein sequencing

Protein bands of interest, collected from ten identical lanes on a one-dimensional mini-SDS/polyacrylamide gel, were excised and in-gel-digested with trypsin, essentially as described in [44]. The resulting peptide mixture was separated on a μ RPC C2/C18 SC 2.1/10 column [SMART (simple modular architecture research tool) system; Amersham Biosciences], developed at a flow rate of 80 μ l/min using an 83 min linear gradient of 0–70 % CH₃CN in 0.1 % trifluoroacetic acid in water. UV-detected (215 nm) peak fractions were analysed by a 492 Procise (PE Applied Biosystems, Warrington, Cheshire, U.K.) amino acid sequencer operating in a pulsed liquid mode.

Cloning, expression and purification of human PME-1

The open reading frame of methylesterase was obtained from EST (expressed sequence tag) clone A1125166 (Human Genome Mapping Project, Hinxton Hall, Cambridge, U.K.) by amplifying the complete methylesterase sequence by PCR using gggaattcccatatgtcggccctcgaaaagagc and ggctcgagatcctaacagccaggaaacacaca as start and stop primers respectively. The PCR fragment was digested with *Bam*HI and *Nde*I and ligated into a *Bam*HI–*Nde*I-digested pet15c vector (Novagen). This plasmid was transformed into BL21/DE3(plysS) bacteria, grown in Luria–

Bertani medium at 37 °C. PME-1 was induced using 0.5 mM isopropyl β -D-thiogalactoside. Bacteria were harvested after an additional 3 h of growth at 37 °C and lysed in 50 mM Tris/HCl (pH 8.0), 150 mM NaCl and 1 mM DTT. His-tagged PME-1 was purified by affinity chromatography on Ni²⁺-agarose beads (Affiland, Liège, Belgium) using 50 mM sodium phosphate (pH 7.4) and 10 mM imidazole as the equilibration and wash buffer, and eluted with the same buffer, supplemented with 250 mM imidazole.

RESULTS

Purification of PP2A methylesterase from porcine brain

The method used for purification of methylesterase from porcine brain was based on a procedure for purification from bovine brain described in [26]. In this procedure, methylesterase was separated from PP2A after an ion-exchange chromatography on DEAE-cellulose during a hydrophobic interaction chromatography (phenyl-Toyopearl). We noticed that, in addition to this 'free' methylesterase eluting at the end of the descending ammonium sulphate gradient on a hydrophobic interaction column (phenyl-Sephacel), most of the methylesterase activity could be eluted from the column, with 20 % glycerol and 50 % ethylene glycol co-eluting with PP2A activity. For further purification, we followed the procedure as outlined in the Materials and methods section and Figure 1(A). PP2A and methylesterase activity could be only partially separated by an ACA gel filtration followed by a polylysine affinity chromatography. An almost complete separation of PP2A and methylesterase activity was achieved by the final FPLC ion-exchange chromatography (Mono Q or Resource Q), as shown in Figure 1(B). A PP2A activity peak (fractions 24–30) contained a trimeric form of PP2A_{T55}, and PP2A methylesterase activity eluted afterwards, apparently well-separated from PP2A. Three proteins, 65, 45 and 36 kDa, co-eluted with methylesterase activity in almost stoichiometric amounts. The 65 and 36 kDa proteins remarkably migrated in SDS/polyacrylamide gel at the same height as the PR65 and C36 of the trimeric PP2A. Western blotting of the individual fractions with monoclonal antibodies against the PR65 and C36 subunits identified these proteins as authentic PR65 and C36 subunits of PP2A (results not shown). From the third protein, running in SDS/polyacrylamide gel as a protein of 45 kDa, three tryptic peptides were isolated and sequenced, namely SIVEGIIEEE, LPSRPPLPGSGG and SMHLG. The three peptides led us to EST clones of sequences with esterase motifs. With overlapping EST clones, we could construct an open reading frame *in silico*. On the basis of the partial sequences in the database and the length of the EST clones, EST clone A1125166 was selected for cloning the complete methylesterase sequence by PCR. The sequence determined by us was identical with the sequence of the esterase (PME-1) associated with the PP2A-inactive mutant forms [27] and will, therefore, be named here after according to this nomenclature, since Stock and co-workers have mentioned in [33] that the sequence of the methylesterase that they purified and named as MEase [26] was identical with PME-1. Also, our sequence can be expressed in bacteria as an active recombinant soluble protein and its His-tagged version can be purified in a one-step affinity purification on Ni²⁺-agarose beads.

PME-1 is associated with an inactive form of PP2A_D or PP2A_{T55}

As seen in Figure 1(B), almost no PP2A activity could be detected in the fractions where PME-1 eluted from the Mono Q column.

Figure 1 Purification of PME-1 and association with an inactive form of PP2A

(A) Diagram showing the steps of purification of PME-1 from porcine brain (see the text for details). (B) Elution profile of PME-1 (◆) and protamine-stimulated phosphorylase phosphatase activity (▲) during the last purification step (Mono Q). One arbitrary unit of PME-1 corresponds to 10 000 c.p.m. [^3H]methanol measured after 60 min under standard assay conditions. Protamine-stimulated phosphorylase phosphatase activity was also measured after a preincubation with PTPA and ATP-Mg^{2+} as described in the Materials and methods section (■). (C) Coomassie Blue-stained 12% SDS/polyacrylamide gel of 7 μl of the indicated fractions.

However, if the fractions were preincubated with PTPA and ATP-Mg^{2+} , a protamine-stimulated phosphorylase phosphatase activity could be measured that was expected for the amount of PR65/C36 protein that was present [compare the activity and the amount of proteins present in peaks 1 (Fractions 26–30) and 2 (Fractions 32–36)]. It is clear that PP2A associated with PME-1 was inactive and its Ser/Thr phosphatase activity could be increased significantly by preincubating with PTPA and ATP-Mg^{2+} . We will refer to this form as PP2Ai hereafter. It is clear that the name phosphotyrosyl phosphatase activator is no longer adequate to cover the function of PTPA as Ser/Thr phosphatase activator, but we suggest retention of the acronym PTPA for historical reasons

and redefine its meaning into phosphatase two A phosphatase activator. From Table 1, it is seen that the amount of PP2Ai that can be purified from brain is considerable and can represent up to 30% of the total amount of PP2A that can be purified from the same tissue, taking into account that nearly the same amount of active PP2A can be purified from pool II of the ACA column as from pool I. Moreover, after activation, the specific activity as calculated for the catalytic subunit that is present is very similar to active PP2A, purified during the same procedure and comparable with the value published previously [40]. From the results shown in Figure 1(C), it is clear that in this preparation, most of the PP2Ai was dimeric PP2A (PP2Ai_D). Nevertheless, there was always a variable amount of a 55 kDa band co-purifying that was immunologically identified as PR55 (Figure 2). The amount of PR55 in PP2Ai varied from preparation to preparation: in some preparations, PR55 was hardly detectable (Figure 1C), whereas in others the amount of PR55 was almost stoichiometric with PME-1 (Figures 2B, 2E and 2F). Moreover, in this case, the phosphorylase phosphatase activity was increased by preincubating with PTPA and ATP-Mg^{2+} , which implies that PP2A_{T55} can also exist as an inactive form of PP2A (PP2Ai_{T55}) associated with PME-1. This was verified by the following experiment: a preparation with a relatively high concentration of PR55 was further purified on a Superdex gel-filtration column and, as seen in Figures 2(C)–2(F), PP2Ai_{T55} co-purifies with PME-1. PP2Ai_{T55} complexed with PME-1 runs with a higher apparent molecular mass in gel filtration compared with the PME-1–PP2Ai_D (pool II) complex. PP2Ai_{T55} was largely inactive and could be considerably activated by PTPA and ATP-Mg^{2+} (Figures 2C and 2F).

Purification of PME-1 and PP2Ai from rabbit skeletal muscles

For purification of PME-1 from rabbit skeletal muscles, a procedure similar to the one described above was used, and very similar results were obtained. Our normal procedure for the purification of PP2A_{T55}, PP2A_{T72} and PP2A_D from rabbit skeletal muscles [40] starts with a batchwise absorption of the crude extract on DEAE-Sephacel, followed by a wash with equilibration buffer and a wash with 0.2 M NaCl in the same buffer. This 0.2 M NaCl wash is a rich source of PME-1 and PP2Ai from which they could be purified as a kind of side-product in the PP2A purification procedure: after precipitation of the 0.2 M NaCl wash with 60% ammonium sulphate, the pellet was solubilized, brought to 0.8 M ammonium sulphate, loaded on to a phenyl-Sepharose column and purified further as outlined in Figure 1(A). The characteristics of PME-1 and the active and inactive forms of PP2A purified from the 0.2 M NaCl wash were very similar to those purified from the 0.15 M NaCl eluate from porcine brain.

PP2A holoenzymes as substrates for LCMT and PME-1

By using a methylation-sensitive antibody in combination with a KOH treatment that fully demethylates PP2A [23], we have shown previously [25] that different PP2A holoenzymes purified from rabbit skeletal muscles are (de)methylated up to different levels. PP2A_D was always fully demethylated, whereas PP2A_{T72} was always fully methylated, and the degree of methylation of PP2A_{T55} varied from preparation to preparation. We next tested the possibility of using these different holoenzymes in the methyl turnover assay and found that the degree of methylation at the start of the assay could almost be neglected: the fully methylated PP2A_{T72} was as good a substrate in the turnover assay as the fully demethylated PP2A_D (results not shown). From Figure 3, it can

Table 1 Purification of PME-1 and the associated PP2A

PME-1 activity is expressed in terms of arbitrary units as indicated in the legend to Figure 1. Protamine-stimulated phosphorylase phosphatase activities (active and inactive) were measured in the pooled fractions at five different dilutions as indicated in the Materials and methods section. Results are the means for at least three different dilutions measured under linear conditions and are expressed as total units. In the chromatographic separation column ACA34, a partial separation was obtained between PME-1 and PP2A, and only pool II with the highest esterase activity was taken further in this scheme. From pool I, only active PP2A could be purified. Also, in the polylysine column, a partial separation of PME-1 and active PP2A was obtained. Two pools were made and both fractions were further purified separately: pool I on Resource Q I and pool II on Resource Q II. As illustrated in Figures 1(B) and 1(C) for a similar purification, an almost complete separation of active PP2A and PME-1 was obtained during this last purification step. NM, not measured.

Purification step	Activity (total units)						Specific activity (units/mg)			
	0.15 M NaCl			0.4 M NaCl			0.15 M NaCl		0.4 M NaCl	
	PME-1	Active PP2A	Inactive PP2A	PME-1	Active PP2A	Inactive PP2A	Active PP2A	Inactive PP2A	Active PP2A	Inactive PP2A
Phenyl-Sepharose	69 320	50 000		120 000	39 900					
DEAE-Sepharose	39 000	8913		72 000	25 300					
ACA										
Pool I	4800	2200		13 200	10 300					
Pool II	14 040	2416		24 200	6200					
Polylysine										
Pool I	6000	312	2188	12 800	1150	3600				
Pool II	3700	2310	NM	4800	3000	NM				
Resource Q I										
Pool I	0	120	0	0	307	0	9300	—	8700	—
Pool II	3000	80	818	6300	184	1656	—	15 700	—	11 900
Resource Q II										
Pool I	200	491	0	0	1125	0	10 400	—	9400	—
Pool II	800	160	798	3300	975	975	—	10 600	—	10 500

be seen that PP2A_{T72} (eluting in fractions 32–37) could not be methylated during its purification by recombinant LCMT, since it was already fully methylated. Indeed, when PME-1 was added together with LCMT and [³H]AdoMet, [³H]methanol was formed in the diffusion assay, indicating that PP2A_{T72} was a substrate for several methylation–demethylation rounds. This would mean that the presence of a third subunit (PR72 or PR55) does not prevent methylation or demethylation. Moreover, we noticed that PP2A_D was always fully demethylated, irrespective of its association with PME-1 (results not shown). In the latter case, it was endogenous PME-1 that prevented stable methylation, but PP2A_{I_D} was clearly a substrate in the methyl turnover assay. When PP2A_D was purified by the ‘classical’ method [40], no PME-1 was detected, and PP2A_D could be used as the substrate for LCMT, as shown previously [25,33]. Neither activation nor inactivation of its phosphorylase phosphatase activity could be observed by methylation. When recombinant active PME-1 was added to active PP2A_D, no inactivation was observed. In addition, LCMT could not reactivate PP2A_I (although stable methylation could not be achieved because of the presence of a high concentration of active PME-1).

Reactivation of PME-1-associated inactive PP2A by PTPA

PTPA was isolated and characterized previously as an *in vitro* activator of the phosphotyrosyl phosphatase activity of PP2A, without influencing the more stable Ser/Thr phosphatase activity [37]. Therefore it is clear that PP2A_I is a novel, previously unidentified form of PP2A. We investigated some characteristics of the activation reaction of PP2A_I by PTPA. Similar to the phosphotyrosyl phosphatase activation reaction [37], the Ser/Thr phosphatase activation reaction also has all the properties of an enzymic reaction: the activation is time- and dose-dependent and is completely dependent on the presence of ATP-Mg²⁺. The concentration of ATP necessary to observe 50% activation was 1.3 ± 0.5 nM in the presence of 5 mM Mg²⁺.

This is approx. 100-fold lower compared with the concentration necessary to activate the PTPase activity of active PP2A_D [37]. In parallel with the activation of the Ser/Thr phosphatase activity of PP2A_I, its PTPase activity (measured using pNPP or tyrosine-phosphorylated RCAM as the substrate) is also activated. There is one significant difference between both reactions: whereas PTPA-induced PTPase activity is deactivated during the phosphatase assay or by removing ATP-Mg²⁺ by gel filtration [37], activation of the Ser/Thr phosphatase activity of PP2A_I is stable and the resulting phosphatase can no longer be distinguished from active PP2A as it is isolated from tissues. It has a similar specific activity, and is also highly stimulated by polycations such as protamine or polylysine.

PTPA/ATP-Mg²⁺ versus Mn²⁺ activation

Previous reports [45–47] have mentioned the inactivation of PP2A by the loss of metal ions, especially after prolonged storage, which could be reversed by Mn²⁺. We made similar observations, not necessarily correlated with the duration of storage or with a particular holoenzyme. However, this Mn²⁺ activation does not seem to be related to activation by PTPA/ATP-Mg²⁺. Although several PP2A_I preparations could be activated by 1 mM Mn²⁺ to the same extent (e.g. the preparation used in Figure 4), or even higher than that by PTPA/ATP-Mg²⁺, others are less activated by Mn²⁺ and more exclusively activated by PTPA/ATP-Mg²⁺ (see e.g. Figure 2F). Also, the experiment shown in Figure 4 clearly demonstrates that both phenomena (activation by Mn²⁺ and PTPA/ATP-Mg²⁺) are distinct: whereas the PTPA/ATP-Mg²⁺-activated PP2A dissociates from PME-1 and remains active after a gel-filtration step, well separated from PTPA, the Mn²⁺-stimulated PP2A (not stimulated by PTPA/ATP-Mg²⁺) remains associated with PME-1 under this condition. This experiment indicates that at least two different PP2A_I species exist, each complexed with PME-1 and that can be activated by its own activator: Mn²⁺ or PTPA/ATP-Mg²⁺. This experiment also

Figure 2 PP2A_{T55} can also associate with PME-1 as an inactive form of PP2A

(A) Elution profile of PME-1 (■) and protamine-stimulated phosphorylase phosphatase (◆) activities during a Resource Q ion-exchange chromatography as the last step in the purification of PME-1 as outlined in Figure 1(A). (B) Coomassie Blue-stained 12 % SDS/polyacrylamide gel of 7 μ l of the indicated fractions. Fractions 37–44 were pooled, concentrated to approx. 2 ml as described in the Materials and methods section and applied to a Superdex-200 16/50 gel-filtration column. (C) Elution profile of the Superdex gel-filtration column of the pool 37–44 of the Resource Q ion-exchange chromatography. Twenty fractions of 2 ml were collected, followed by fractions of 1 ml. Methylesterase (■) and protamine-stimulated phosphorylase phosphatase activities after activation by PTPA/ATP-Mg²⁺ (◆) were measured in the indicated fractions as described in the Materials and methods section. (D) The indicated fractions (7 μ l) were subjected to SDS/PAGE (12 % gel), Coomassie Blue-stained, extensively destained and then silver-stained. We noticed that the PR55 band was much less silver-stained compared with PR65 and PME-1. (E) The same fractions were blotted in parallel on a Hybond-P membrane (Amersham Biosciences), which was developed using anti-PR55 and anti-PME-1 antibodies consecutively. Positions of PR65 and C36 as immunologically confirmed by Western-blot analysis are indicated by arrows. Two pools (38–43 and 44–49) were made, concentrated by consecutive dialyses against poly(ethylene glycol) and 60 % glycerol and again subjected to SDS/PAGE and Coomassie Blue staining (inset in F). (F) Protamine-stimulated phosphorylase phosphatase activity was measured in the concentrated pools I and II in four different dilutions (1:10 to 1:160) in the presence and absence of protamine after a 10 min preincubation at 30 °C in the presence of PTPA, ATP-Mg²⁺ or 1 mM MnCl₂ or the control buffer. The results of these linear assays as a function of the dilution were calculated for the undiluted enzyme and the S.E.M. are indicated. prot, protamine.

demonstrates that PME-1 has a much higher affinity for PP2A_i than for active PP2A: once activated, PP2A no longer binds to PME-1 (see Figure 4).

Figure 3 PP2A_{T72} is a substrate for PME-1

The last purification step in the purification of PP2A_{T72} is a Mono Q column (40), wherein the remaining PP2A₀ (fractions 27–31) is separated from PP2A_{T72} (fractions 32–37). Each indicated fraction was assayed for protamine-stimulated phosphorylase phosphatase activity (◆) and its suitability as a substrate for added recombinant LCMT and [³H]AdoMet (▲) or as a target for methylation–demethylation in the [³H]methyl turnover assay by adding recombinant PME-1 (■) was tested.

Figure 4 PTPA/ATP-Mg²⁺ and Mn²⁺ activations are different

A PP2A_i preparation (50 μ l), which could be stimulated by Mn²⁺ and PTPA/ATP-Mg²⁺ equally well, was subjected to gel filtration on two Superdex-200 HR 10/30 columns. The first column was loaded with the untreated preparation (A), whereas the second was loaded with a preparation treated with PTPA/ATP-Mg²⁺ (B). Seven fractions of 1 ml were collected, followed by fractions of 200 μ l. The indicated fractions were assayed for protamine-stimulated phosphorylase phosphatase activity without pretreatment (▲) or after activation with PTPA/ATP-Mg²⁺ (◆) or Mn²⁺ (■). PME-1 activity was measured and it co-eluted with the Mn²⁺-stimulated activity in both cases (results not shown). PTPA activity was measured in the fractions of (B) and it eluted as a monomeric protein of 40 kDa with its maximum in fraction 54 (results not shown).

DISCUSSION

In the present study, we purified a PP2A-specific methylesterase that co-purifies with PP2A and identified it as PME-1, and we

found that it was tightly associated with an inactive form of PP2A that can be reactivated by PTPA/ATP-Mg²⁺. PTPA was characterized previously as an activator of the intrinsic tyrosyl phosphatase activity of PP2A, and it is shown here to activate also the Ser/Thr phosphatase activity of the inactive PP2A-PME-1 complex. Therefore we suggest changing the expansion of the acronym PTPA to phosphotyrosyl phosphatase two A phosphatase activator.

Dynamic regulation of the composition of PP2A is to be the underlying paradigm of its involvement in many of its physiological functions, and methylation of PP2A_C at its well-conserved C-terminus was suggested to influence the binding of third regulatory subunits [31–36]. Moreover, it was demonstrated that exogenously added PR61, and to a lesser extent PR55, inhibit the demethylation of methylated PP2A_D [33]. In addition, it was shown that the isolated ³H-methyl-labelled PP2A_{T61} (AB'C) is much more resistant to demethylation by PME-1 when compared with the ³H-methyl-labelled PP2A_D (AC), and it was concluded that 'holoenzyme formation essentially locks in the methyl group for the lifetime of the complex' [33]. As a consequence, this would substantially impair reversible methylation as the dynamic force for a differential composition of PP2A. In the present study, we demonstrate that the presence of a third subunit (PR72 or PR55) does not prevent methylation or demethylation. This is more in line with the results presented by the same authors [33] who mentioned that the fully methylated PP2A_{T61} (AB'C) was radiolabelled by adding [³H]AdoMet to crude extracts, implying that this holoenzyme was first demethylated and remethylated afterwards. Taken together, these results show that methylation of PP2A holoenzymes is a reversible process and, as a consequence, these oligomers are available for the exchange of third subunits.

The determinants on PP2A for PME-1 binding are not known, but it is intriguing to note that two inactive mutant forms of PP2A_C also bind to PME-1 [27]. In these mutants, two histidine residues (His⁵⁹ and His¹¹⁸), believed to be involved in catalysis and substrate binding respectively [27], were individually mutated into glutamine residue and shown to be catalytically inactive [27,30,48]. It is tempting to speculate that PP2A_i, and the mutants H59Q (His⁵⁹ → Gln) and H118Q, would have a similar inactive conformation that would allow binding to PME-1. It was argued that PME-1 binding to PP2A was partially to the C-terminus of PP2A (the substrate site for PME-1) and partially to the vicinity of the catalytic site: active-site residues themselves and/or metals, or nearby residues [27]. The fact that PR55 and PME-1 binding are not mutually exclusive is not so surprising. The catalytic site of PP2A is still accessible even for large substrates such as phosphorylase, even in the trimeric holoenzyme. If PME-1 indeed binds on to, or in the proximity of, the catalytic site, PR55 will not be a factor of steric hindrance. The same arguments are in principle valid for all the other 'third' subunits. The fact that PP2A_{T55} can also exist, at least partially, as an inactive PP2A that can be reactivated by PTPA indicates that PR55 is neither preventing the activation by PTPA nor the inactivation by a factor that still needs to be discovered or that might be PME-1 itself (see below). Since the inactivation–reactivation cycle is not limited to the dimeric enzyme, it seems to be a general mechanism, unrelated to the subunit composition of PP2A, although we cannot exclude the possibility that the other trimers are more resistant to inactivation.

The mechanism that converts active PP2A into its PTPA/ATP-Mg²⁺-dependent enzyme form PP2A_i has not yet been identified. Since PP2A_i was isolated from porcine brain and rabbit muscles in a tight complex with PME-1, one might speculate whether PME-1 would inactivate PP2A. However, several observations argue against demethylation as being the mechanism: (i) demethylated

PP2A_D exists as an active phosphatase; (ii) reactivation requires PTPA/ATP-Mg²⁺ and not methylation, since methyltransferase cannot reactivate the inactive enzyme form; and (iii) active PP2A_{T55} that co-purifies a long way with the inactive complex is not inactivated during the purification (Figure 1). The finding of a methyltransferase associated with PP2A suggests an additional role for this protein in the regulation of PP2A. Since demethylation as such does not automatically lead to the formation of dimeric phosphatase and does not result in the inactivation of phosphatase activity (demethylated active PP2A_{T55} exists; see [25]), we consider the association of PP2A_{iD} or PP2A_{iT55} with PME-1 as a stabilizing factor after PP2A inactivation by (an)other still unknown mechanism(s). What could be this mechanism? Recently, we demonstrated that PTPA has a peptidyl prolyl-*cis/trans*-isomerase activity (J. Jordens, V. Janssens, I. Stevens, E. Martens, Y. Engelborghs, E. Waelkens, J. Goris and C. van Hoof, unpublished work), probably responsible for a conformational change of PP2A. Therefore we speculate that the inactivation is also caused by a conformational change, but now in the opposite direction. This would result in a PTPA/ATP-Mg²⁺-dependent PP2A, which in addition might easily (but not necessarily) lose its Zn²⁺ and Fe²⁺ and become Mn²⁺-dependent. It has been shown previously [46,47] that active PP2A is a Zn²⁺- and Fe²⁺-containing metalloenzyme that becomes Mn²⁺-dependent by losing its metal ions. We speculate that the Mn²⁺-dependent PP2A_D that was detected in several of our PP2A_i preparations lost its metal ions. In contrast, PP2A_i can be activated by PTPA/ATP-Mg²⁺ without the addition of Zn²⁺ or Fe²⁺ and, therefore, it is supposed to still contain both the metal ions. Once activated by PTPA/ATP-Mg²⁺, PP2A dissociates from PME-1 (Figure 4). Since both the Mn²⁺- and PTPA/ATP-Mg²⁺-dependent forms of PP2A_i co-purify and have apparently similar affinities for PME-1, they probably have a very similar conformation. We suggest that PP2A in its PP2A_i conformation is prone to lose its metal ions. Once this occurs, PP2A can only be reactivated *in vitro* by rather high Mn²⁺ concentrations (1 mM versus the cellular Mn²⁺ concentration, which is estimated, for instance, in liver to be 1.200 µg/kg [49] or 14 µM if one assumes 50% for the soluble compartment). Therefore the cell is believed to prevent this (irreversible) loss of metal ions from PP2A. Activation by PTPA/ATP-Mg²⁺ is certainly a mechanism that would prevent this loss of PP2A activity and it is only when PTPA is absent or diminished that PP2A might (irreversibly) be converted into its Mn²⁺-dependent apoenzyme. It is possible that separation of PTPA during the purification might contribute to an inactivation; however, this fact on its own cannot explain the generation of PP2A_i, since PTPA is separated from PP2A early in the purification (it elutes at a very low salt concentration on a DEAE-cellulose/Sephacel gel matrix [38,39]) and no PTPA can be detected in the final PP2A preparations (results not shown). Nevertheless, most of these preparations are active. It is clear that only a selected pool of PP2A is, or becomes, inactive. At the moment, we are not able to distinguish between the possibility that PP2A is synthesized in an inactive conformation that needs to be activated by PTPA acting as a kind of chaperone, or that PP2A_i is indeed actively formed by an 'inactivating principle'.

Other mechanisms of PP2A inactivation have been described. Phosphorylation of PP2A_C at Tyr³⁰⁷ [12,13] or at an unidentified threonine residue [15] have been described to inactivate PP2A. However, any speculation on phosphorylation being the sole underlying mechanism of inactivation is doubtful, since inactivation by tyrosyl phosphorylation at Tyr³⁰⁷ as well as by phosphorylation at an undefined threonine residue are known to be reversed by autodephosphorylation and no (spontaneous) activation of PP2A_i is ever observed.

The fact that PP2Ai could be purified from mammalian tissues and binds to PME-1 as did two inactive synthetic mutants of PP2A supports the hypothesis that such an enzyme form would exist *in vivo*. Further support can be deduced from the functional analysis of PTPA in *Saccharomyces cerevisiae*: PTPA is an essential gene and deletion of both *YPA1* and *YPA2* (the PTPA homologues in yeast) is lethal [50–52]. A genetic interaction of PP2A and PTPA was demonstrated previously [51] and it was shown that PTPA could influence the relative proportion of trimeric and dimeric forms [51]. Moreover, PTPA seems to influence only a subset of PP2A functions [51]. Since phosphorylation on tyrosine residues is a rare event in yeast [53], the function of PTPA as an activator of the Ser/Thr phosphatase activity of an inactive pool of PP2A would be a more probable and possibly the only function in yeast.

When this work was in a final stage, we came across the work of Ogris and co-workers [54]. By studying the substrate specificity of PP2A in a yeast strain with a double deletion of *YPA1/RRD1* and *YPA2/RRD2*, still viable owing to its *SSD1-v*-positive genetic background (where *SSD1-v* is the viable allele of the polymorphic gene *SSD1*), the authors found a decreased Ser/Thr phosphatase activity of PP2A that could be stimulated by Mn^{2+} or by reintroduction of *Ypa2/Rrd2* up to the wild-type activity level. Loss of PTPA function caused the synthesis of a less stable PP2A having an increased pNPPase activity. It was concluded that PTPA was probably essential for the right conformation of PP2A for its Ser/Thr phosphatase activity. In the absence of PTPA, PP2A seems to be converted into an inactive apoenzyme, which can be reactivated by Mn^{2+} . After reintroduction of *Ypa2/Rrd2*, the same PP2A activity was found as in wild-type PP2A. Also, our results favour the existence of an inactive form of PP2A that can be fully activated by PTPA/ATP- Mg^{2+} . However, our results also indicate that PP2Ai can be discriminated from the Mn^{2+} -stimulated enzyme form. Although both enzyme forms are very similar, they are not identical.

The existence of an inactive PP2A has far reaching consequences for our understanding of PP2A regulation. Since the activity can be recovered by PTPA/ATP- Mg^{2+} activation, it is tempting to speculate that also an inactivating mechanism exists *in vivo*, providing a novel regulatory circuit of PP2A, in addition to the existing multitude of complex regulatory devices of PP2A. It will be interesting to determine whether and how the relative amounts of inactive PP2A would change in response to extracellular signals or different cellular conditions, such as cell-cycle growth and differentiation.

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