Partial purification and characterization of a type 1 protein phosphatase in purified nuclei of pea plumules

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We report the isolation and characterization of a protein Ser/Thr phosphatase from highly purified pea nuclei. In subnuclear fractions, more than 75% of Ser/Thr protein phosphatase activity was associated with the chromatin fraction, whereas the other 25% was in the nuclear membrane/nucleoplasmic fraction when phosphorylase *a* was used as a substrate. The enzyme was purified approx. 2750-fold to a specific activity of approx. 4000 nmol/min per mg. The molecular mass of the enzyme was 34 kDa as estimated by molecular sieve chromatography, and approx. 40 kDa as estimated by SDS/PAGE. The phosphatase was inhibited by okadaic acid with an IC_{50} of approx. 15 nM, by

rabbit muscle inhibitor 2 with an IC_{50} of approx. 10 nM, and by microcystin-LR with an IC_{50} of approx. 0.05 nM. The enzyme did not require Ca^{2+} , Mg^{2+} or Mn^{2+} for its activity; instead, these cations showed some inhibitory effects. It was inhibited by NaF or citrate but not by tartrate, molybdate or vanadate under the conditions tested. Its sensitivities towards the various phosphatase inhibitors and its substrate specificity were very similar to those characteristic of the type 1 Ser/Thr protein phosphatases well studied in animal systems. The enzyme was able to selectively dephosphorylate a 92 kDa nuclear protein that had been phosphorylated by one or more endogenous protein kinases.

INTRODUCTION

Phosphatases that dephosphorylate proteins at Ser and Thr residues are generally classified as type 1, type 2A, 2B and 2C (PP1, PP2A, PP2B and PP2C) according to their substrate specificity, ion requirement and sensitivity to inhibitors [1]. Both PP1 and PP2A are active in the absence of divalent cations, whereas PP2B and PP2C have an absolute requirement for Ca^{2+} and Mg^{2+} respectively. Both PP1 and PP2A can be inhibited by okadaic acid, but with different sensitivities: the inhibition of PP2A is complete by 1–2 nM but PP1 is hardly affected at this concentration, its complete inhibition requiring 1 μ M okadaic acid. These two phosphatases can be further distinguished by two heat-stable protein inhibitors, inhibitor 1 and inhibitor 2, which block the activity of PP1 but not PP2A [1]. Recently several novel Ser/Thr-type protein phosphatases have been identified that do not fit into the above classification; however, this classification is still broadly applicable and remains widely used [2].

The catalytic subunits of PP1 and PP2A associate with regulatory subunits that may control their activity, substrate specificity and/or targeting to particular subcellular locations. In animal cells, PP1 and PP2A were initially isolated from the cytosolic fraction. Later they were also found in the nucleus [3–6]. Recently Chen et al. [7] isolated a cDNA encoding a novel human Ser/Thr protein phosphatase that localizes to the nucleus. It has a long N-terminal domain containing four tetratricopeptide repeats similar to those found in several nuclear proteins. Thus it appears that the nuclear location of protein phosphatases might be controlled either by their regulatory subunits or by their own nuclear localization signal sequences in the catalytic subunits. Both PP1 and PP2A are known to be involved in many

nuclear processes, such as mitosis, RNA biogenesis, transcription regulation and nuclear transport [1,3].

In comparison with protein phosphatases from animal cells, much less is known about the biochemical characterization, localization and function of those in plants. Early studies of plant protein phosphatases did not try to compare their biochemical and enzymic properties with those of animal protein phosphatases [8–10]. Since then, several reports have documented the presence of phosphatase activities in plants resembling those reported for PP1, PP2A and PP2C in animal cells [11–13]. The plant PP2A might regulate the activities of enzymes such as quinate dehydrogenase [12], sucrose-phosphate synthase [14], nitrate reductase [15] and phosphoenolpyruvate carboxylase [16]. The antagonists okadaic acid and microcystin-LR have been used to demonstrate that PP1 and PP2A phosphatases are involved in processes such as pollination [17], defence responses [18] and light-inducible gene expression changes in maize [19]. In these inhibitor studies, information about the biochemical and enzymic properties of the inhibited protein phosphatases themselves is very limited. However, cDNAs encoding protein phosphatases similar to PP1 and PP2A in animal cells have been isolated from several plants [20–24]. The remarkable similarity in the sequences of these cDNAs to those of the cDNAs encoding animal phosphatases, together with the similarity in sensitivity to phosphatase inhibitors of animal and plant PP1 and PP2A, indicates that they may play similar essential roles in the regulation of metabolism.

There is little information available on the subcellular localization of protein phosphatases in plant cells. MacKintosh et al. [12] reported that PP1 and PP2A are found in either particulate or soluble fractions depending on the species, but their exact subcellular localizations are unknown. Phosphatases of the PP1

Abbreviations used: CDPK, Ca^{2+} -dependent protein kinase; CK-2, casein kinase 2; IEF, isoelectric focusing; PP1, PP2A, PP2B, and PP2C, type 1, type 2A, 2B and 2C Ser/Thr protein phosphatase respectively; PP1c, type 1 Ser/Thr protein phosphatase catalytic subunit; PTPase, protein tyrosine phosphatase; PKA, cAMP-dependent protein kinase.

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and PP2A types (as defined by inhibitor criteria) were not detected in the membranes or stromata of chloroplasts of plants examined [12,25], nor was any other bivalent-cation-dependent or -independent protein phosphatase found to be specifically associated with chloroplasts [12]. A histone phosphatase previously reported to be associated with chloroplasts proved to be a contaminant enzyme from other cellular compartments [10,25]. None the less, it is well known that protein phosphorylation/dephosphorylation is involved in the regulation of some processes in the chloroplasts [26]. It remains to be discovered what type(s) of protein phosphatases are responsible for the dephosphorylation in those processes.

Datta et al. [27] found that the irradiation of pea nuclei with red light could stimulate the phosphorylation of several nuclear proteins. After this initial discovery, a Ca^{2+} -dependent protein kinase (CDPK) and casein kinase 2 (CK-2)-type protein kinases were purified and characterized from isolated pea nuclei [28,29]. These results indicated that protein phosphorylation and dephosphorylation are important in the regulation of nuclear function. However, the protein phosphatases responsible for the dephosphorylation of nuclear proteins are essentially unknown.

As part of our effort to understand the role of phosphorylation and dephosphorylation in plant nuclear metabolism, we have begun to investigate the properties of nuclear protein phosphatases. Initially we detected both protein Ser/Thr phosphatase and Tyr phosphatase (PTPase) activities in a chromatin-enriched fraction of isolated pea nuclei. The enzyme with PTPase activity was partly purified and characterized [30]. Preliminary evidence indicates that the Ser/Thr phosphatase is a PP1-like phosphatase. This conclusion is confirmed by the further studies reported in this paper. Here we present the first evidence that a PP1-type protein phosphatase is present in plant nuclei as it is in animal cells, and we describe its general biochemical and enzymic properties and its ability to dephosphorylate endogenous nuclear phosphoprotein(s).

EXPERIMENTAL

Plant materials

Plumules were harvested under green light from seedlings of pea (*Pisum sativum* L. cv. Alaska) that had been grown in the dark for 7 days at $23 \pm 3^\circ\text{C}$.

Chemicals

Aprotinin (from bovine lung), phosphorylase *b*, phosphorylase kinase (from rabbit muscle), dephosphorylated casein, histone IIA, histone IIIS (from calf thymus), histone IIA-agarose, okadaic acid, Percoll and PMSF were from Sigma. Microcystin-LR was from Calbiochem. All buffers were prepared with water purified by a MilliQ water purification system (Millipore).

Isolation of nuclei and preparation of subnuclear fractions

All procedures were performed at 4°C except the HPLC chromatographies, which were run at room temperature ($23 \pm 3^\circ\text{C}$). Nuclei were isolated from the plumules of dark-grown pea seedlings as previously described [27]. Routinely, the nuclei isolated from 100–200 g of pea plumules were used as starting material. The nuclei were lysed by incubating them in buffer A [60 mM Hepes (pH 7.5)/1 mM EDTA/0.5% (v/v) Triton X-100/0.5 mM PMSF/0.5% (v/v) aprotinin/0.1% 2-mercaptoethanol] for 10 min at 4°C , and then centrifuged for 2 min. All centrifugations were at 9000 *g* unless otherwise noted. The supernatant was designated as the Triton X-100 fraction and

the pellets as the chromatin fraction; the latter included nuclear matrix and nucleoskeletal elements as well as chromatin.

The chromatin fraction was briefly washed with buffer B [60 mM Hepes (pH 7.5)/3 mM MgCl_2 /0.5 mM PMSF/0.5% (v/v) aprotinin/0.1% 2-mercaptoethanol] and centrifuged. The pellet was extracted with buffer B plus 0.35 M NaCl by stirring it for 30 min and then centrifuging it for 5 min. The supernatant was saved and the chromatin pellet was extracted again with the same buffer. The supernatants from the two extractions were combined and referred to as the 0.35 M NaCl extract. The pellet was extracted again by stirring it with buffer B plus 1 M NaCl followed by a 10 min centrifugation. The supernatant was termed the 1 M NaCl extract and the pellet discarded.

Purification of the phosphatase

The 0.35 M NaCl extract was used as the source for the purification of the phosphatase. It was diluted with buffer C [25 mM Tris/HCl (pH 7.5)/0.5% (v/v) aprotinin/0.5 mM PMSF/0.1% 2-mercaptoethanol] to a final concentration of 0.1 M NaCl in the buffer, then clarified by centrifugation for 15 min. The supernatant was loaded on a histone IIA-agarose column (1 cm $\times$ 6 cm) that had been previously equilibrated with buffer C at a flow rate of 0.5 ml/min. The column was washed with buffer C containing 0.1 M NaCl and eluted stepwise with buffer C containing 0.3, 0.6 and 0.9 M NaCl at a flow rate of 0.5 ml/min. The active fractions were found in the 0.3 M NaCl elution. They were collected and combined, concentrated by an Amicon Centricon-10 concentrator, and applied to an HPLC molecular sieve column (G3000 SW; 30 cm $\times$ 7.8 mm; Supelco), equilibrated and eluted with buffer D [25 mM Tris/HCl (pH 7.0)/200 mM NaCl/1 mM EDTA/1 mM EGTA/10% (v/v) glycerol]. The flow rate was 0.5 ml/min; 0.25 ml fractions were collected. The active fractions were collected and loaded on a HEMA IEC BIO DEAE column (7.5 mm $\times$ 7.5 cm; Alltech Associates) that had been equilibrated with buffer E [25 mM Tris/HCl (pH 7.0)/1 mM EDTA/1 mM EGTA/10% (v/v) glycerol]. After unbound proteins had been washed off the column with buffer E, the bound proteins were eluted with a linear NaCl gradient (0–0.9 M) in buffer E in 30 min. The active fractions were combined and refractionated by the same HPLC molecular sieve column under the same conditions. The active fractions were used for the characterization analyses described below unless otherwise indicated.

Determination of molecular mass and isoelectric point of the phosphatase

The native molecular mass of the phosphatase was estimated by a comparison of retention times between active fractions and molecular mass standards eluted on the HPLC molecular sieve column as described above.

The pI of the protein phosphatase was determined by isoelectric focusing (IEF) agarose gel electrophoresis as described by Guo and Roux [30]. A portion of partly purified phosphatase sample taken either from the DEAE column or from the second molecular sieve column was concentrated by Microcon-10. For the sample from the second molecular sieve column, approx. 100 μg of cytochrome *c* was added as a carrier protein. The concentrated sample was subjected to IEF on an analytical IEF gel (pH range 3–10; IsoGel FMC). When the focusing was complete, the gel slab was cut into 2 mm slices, which were extracted with 25 mM Tris/HCl (pH 7.0)/0.1% 2-mercaptoethanol/1 mM EDTA/1 mM EGTA. The extract was assayed for phosphorylase *a* phosphatase activity under standard conditions except that the substrate (phosphorylase *a*) concentration

was 5 μ M and the incubation time was 20 min. The pI of the phosphatase was determined by the pI markers run on the same gel plates. The extract from active slices was added to SDS sample buffer and run on SDS/PAGE to determine the molecular mass of the protein present.

Preparation of 32 P-labelled substrates

32 P-labelled histone IIA and casein were prepared by phosphorylation with a partly purified catalytic subunit of cAMP-dependent protein kinase (PKA) from reticulocytes by the method described by Grankowski et al. [31]. 32 P-labelled rabbit skeletal muscle phosphorylase *a* was prepared as described by Cohen et al. [32]. The specific radioactivity was approx. 10^5 – 10^6 c.p.m./nmol. Histone IIS and casein were phosphorylated on Ser/Thr residues by pea nuclear protein kinases, histone by CDPK, and casein by a CK-2. The CDPK and CK-2 were partly purified by a DEAE-5PW HPLC column step as described previously [28,29]. Phosphorylated proteins were precipitated by 20% (w/v) trichloroacetic acid. The pellet was washed with a mixture of ethanol/ether (1:1, v/v), then dissolved in 1 M Tris/HCl, pH 7.5, and dialysed, first against deionized water for 24 h (with three changes), then against 25 mM Tris/HCl, pH 7.0, overnight.

Phosphorylation of nuclear proteins by endogenous protein kinases was performed by two methods. In the first method the phosphorylation reaction was conducted with highly purified intact nuclei as described by Datta et al. [27], with some modifications. After the phosphorylation reaction, the nuclei were collected by centrifugation, and the nuclear proteins associated with the chromatin fraction were extracted with buffer B containing 0.35 M NaCl as described earlier. In the second method the phosphorylation reaction was conducted in the 0.35 M NaCl chromatin extract, which was diluted to a final NaCl concentration of 0.1 M. CaCl_2 and MgCl_2 were added to the solution to a final concentration of 0.5 and 5 mM respectively.

NaF (5 mM) and sodium vanadate (0.1 mM) were also included in the reaction mixture. The reaction was initiated by adding [γ - 32 P]ATP. In both cases the nuclear proteins were precipitated by adding trichloroacetic acid to a final concentration of 10% (w/v). The pellet was washed and redissolved as described above, then dialysed extensively against water and freeze-dried. The dried protein sample was redissolved in 25 mM Tris/HCl (pH 7.0)/100 mM NaCl/1 mM EDTA/1 mM EGTA and clarified by centrifugation. The supernatant was used as the source of nuclear phosphoproteins for the dephosphorylation reactions.

Phosphatase activity assays

The protein phosphatase activity was determined by measuring the release of 32 P from 32 P-labelled substrates. The reaction mixture contained 10 μ l of buffer stock (125 mM Mes/NaOH, pH 6.5), 0.1% 2-mercaptoethanol, 10 μ l of 32 P-labelled histone IIA or casein in a buffer of 25 mM Tris/HCl, pH 7.0, or phosphorylase *a* in a buffer of 25 mM Tris/HCl (pH 7.0)/0.1 mM EDTA/0.1% 2-mercaptoethanol/15 mM caffeine with 10 μ l of enzyme preparation from various purification stages diluted with buffer D containing 1 mg/ml BSA. The final concentration of substrates was 2–5 μ M for histone IIA or casein, and 10 μ M for phosphorylase *a*. For the reactions with phosphorylase *a* or casein as substrates, the reaction was initiated by adding the substrate, then maintained at 30 °C for 10 min, and stopped by the addition of 100 μ l of 20% (w/v) trichloroacetic acid and 10 μ l of 25 mg/ml BSA as a carrier protein. The mixture was vortex-mixed, kept at room temperature for 2 min, then centrifuged for 5 min. A 120 μ l aliquot of the supernatant was added

to 2 ml of aqueous scintillant and counted in a Packard scintillation counter. Blank incubations were performed in which the enzyme preparations were replaced by the buffer. For reactions with histone IIA as the substrate, two assay methods were used. The first method was essentially the same as for phosphorylase *a* except that the incubation period for the reaction was 20 min. In the second method the phosphate released was extracted with butanol/toluene (1:1, v/v) after formation of the phosphomolybdate complex as described [8] with some modifications. The enzyme preparation was diluted so that dephosphorylation of the substrate was kept under 30% of the total radioactivity. The above assay conditions are referred to below as standard conditions. One unit of enzyme activity was defined as the amount that released 1 nmol of phosphate/min. The various agents tested were added to the reaction mixture to the final concentrations as indicated and preincubated at room temperature for 10 min before the reaction was initiated with substrate. The dephosphorylation of casein phosphorylated by pea CK-2 kinase was performed in similar way to that described above. For the dephosphorylation of histone IIS phosphorylated by pea nuclear CDPK, and pea nuclear proteins phosphorylated by the endogenous nuclear protein kinases, the reaction was stopped by adding SDS sample buffer. The dephosphorylation was monitored by autoradiography after SDS/PAGE.

Other methods

Protein concentration was determined with a Bio-Rad protein assay kit with BSA as a standard unless otherwise indicated. SDS/PAGE was by the method described by Laemmli [33].

RESULTS

Distribution of the extractable protein phosphatase activities in subnuclear fractions

The purity of the nuclear preparation was judged by the methods described in Guo and Roux [30]. Phosphatase activity was extracted from nuclei sequentially by using first Triton X-100, then 0.35 M NaCl, then 1 M NaCl, yielding 26%, 65% and 9% respectively of the total extractable phosphatase activity with phosphorylase *a* as substrate. Very little phosphatase activity remained associated with the chromatin–matrix residue after these extractions (results not shown). A small fraction of the phosphatase activity extracted by 0.35 M salt precipitated out of solution when this extract was diluted before being loaded on the histone IIA–agarose column. This precipitate was removed by centrifugation, and so only those nuclear phosphatases stable in the loading buffer for the first column were subjected to further purification.

Purification of the phosphatase

After each purification step the phosphatase activity was routinely monitored by using histone IIA as substrate (method 1) because it is inexpensive and convenient to label. This method gave a high background owing to the incomplete precipitation of histone by trichloroacetic acid, yet the results were consistent and repeatable. Method 2 was used for more accurate determination of the specific activity. At each chromatographic purification step, the histone phosphatase activity was eluted as a single peak (Figure 1). The fractions from each purification step were also checked for phosphorylase *a* phosphatase activity, and both activities were found to be eluted at the same positions. The final enzyme preparation from the second molecular sieve column was not homogeneous as judged by SDS/PAGE. It

Figure 1 Elution profiles of A_{280} and phosphatase activity from (A) first molecular sieve column, (B) DEAE column and (C) second molecular sieve column

The enzyme activity was assayed with histone IIA as the substrate. The molecular mass markers used to calibrate the molecular sieve column in (C) were: thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). Each point represents the average of duplicate assays obtained under standard conditions. The inset in (C) shows silver-stained proteins from the active fractions of the second molecular sieve column after SDS/PAGE.

Table 1 Purification of PP1 from pea nuclei

The nuclei isolated from 200 g of pea plumules were used as starting material. The activity was assayed in duplicates with phosphorylase *a* as substrate.

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (fold)
Chromatin extract	48.00	69.6	1.5	1
Histone-agarose column	7.00	20.4	2.9	2
Molecular sieve column (1)	0.63	17.3	27.4	19
DEAE column	0.13	11.3	90.4	62
Molecular sieve column (2)	0.002*	6.0	3991.0	2752

* Estimated from the silver-staining intensity of protein band after SDS/PAGE.

seemed to contain four major protein components (at 58, 40, 35 and 28 kDa) as judged by silver staining (Figure 1C, inset).

The purification achieved at each step is summarized in Table 1. The enzyme was purified 2752-fold with a final specific activity of approx. 4000 nmol/min per mg when phosphorylase *a* was

Figure 2 Determination of pI and molecular mass after IEF gel electrophoresis

An enzyme sample concentrated from the most active fraction of the DEAE column was fractionated by IEF. The lanes of the focused gel were cut into 2 mm slices, and the proteins extracted from each slice were tested for phosphatase activity. The extracts from the gel slices were fractionated by SDS/PAGE and stained with silver stain. The stained protein band from the two most active IEF gel slices (34 and 35) is shown in the inset.

used as substrate, and 10 nmol/min per mg when histone IIA was used as substrate under the conditions specified. This enzyme preparation was used for the characterization studies unless otherwise indicated.

Molecular mass and isoelectric point of the phosphatase

The native molecular mass of the phosphatase was approx. 34 kDa on the basis of molecular sieve column chromatography (Figure 1C). The enzyme preparation after the final column step was not homogeneous as judged by SDS/PAGE. This preparation could be further fractionated by IEF gel electrophoresis to identify the protein band(s) responsible for the phosphatase activity. When a partly purified enzyme sample was fractionated on an IEF gel, the activity was found in the gel slices corresponding to pI values of 5.4–5.5 (Figure 2). The protein bands were not visible on the IEF gel with Coomassie Blue staining, but when the protein extracted from the gel slices of the IEF gel that contained phosphatase activity was analysed by SDS/PAGE, only a single protein band was detectable by silver staining of the gel. The two slices with the most activity contained the same protein band of 40 kDa (Figure 2, inset), indicating that this protein was most probably responsible for the protein phosphatase activity. The protein band at 40 kDa represented approx. 20% of the protein revealed by silver staining in the sample used for IEF and for the various phosphatase assays (Figure 1C, inset). The same result was obtained whether the peak-purity enzyme preparation from the DEAE column or the preparation from the second molecular sieve column was used for the IEF separation.

Substrate specificity and pH optimum

Among the phosphoproteins tested, phosphorylase *a* was the most efficiently dephosphorylated by the phosphatase. The histone IIA that had been phosphorylated by PKA was also dephosphorylated, but with a much lower specific activity, namely 1/400 that of the phosphorylase *a* phosphatase activity. The dephosphorylation of casein phosphorylated by PKA was negligible. Histone IIS phosphorylated by pea nuclear CDPK

Figure 3 Effects of phosphatase inhibitors on pea nuclear PP1 activity

The activities are expressed as a percentage of that of control samples, which were assayed in the absence of inhibitors. The enzyme assay was performed in duplicate and each point represents the average of two separate experiments.

Table 2 Effects of various agents on the nuclear PP1 activity

The activity assay in each experiment was performed in duplicate under the standard conditions. The reaction mixture contained 0.3 mM EDTA and 0.3 mM EGTA in addition to the agent being tested. Similar results were obtained in three separate experiments.

Agent	Concentration (mM)	Relative activity (%)
None	5	100
CaCl ₂	5	64
MgCl ₂	5	46
MnCl ₂	5	34
NaF	5	2
Citrate	5	8
Tartrate	5	78
NH ₄ molybdate	0.1	112
Vanadate	0.1	120

Figure 4 Effect of NaCl on pea nuclear PP1 activity

The activity is expressed as a percentage of that of a control sample, which contained 5 mM NaCl in the assay buffer. The enzyme assay was performed in duplicate and each point represents the average of two separate experiments.

and casein phosphorylated by pea CK-2 could also be dephosphorylated. The pH optimum was approx. 7.0 when phosphorylase α was the substrate. A similar pH-dependent profile was obtained when histone IIA was used as the substrate.

Effects of phosphatase modulators

Various agents that are known to have effects on phosphatase activity (here called phosphatase modulators) were tested for their effects on the pea nuclear phosphatase with phosphorylase α as substrate. The phosphatase was inhibited by okadaic acid, a potent protein inhibitor of PP1 and PP2A, with an IC_{50} of approx. 15 nM (Figure 3A), a sensitivity level that indicates that this protein phosphatase is probably a PP1 type. This view is confirmed by the fact that the enzyme could also be completely inhibited by inhibitor 2, a specific PP1 inhibitor identified in animal cells, with an IC_{50} of approx. 10 nM (Figure 3B). The sensitivities of the pea phosphatase towards okadaic acid and inhibitor 2 were at levels similar to those reported for animal [5] and plant PP1 [11]. Microcystin-LR, another potent PP1 (and PP2A) phosphatase inhibitor, could completely inhibit the pea phosphatase at a concentration of 1 nM (Figure 3C), similarly to its effects on mammalian PP1 and PP2A [7].

The effects of ions known to regulate certain phosphatase activities on the pea nuclear phosphatase are summarized in Table 2. The enzyme does not require Ca^{2+} , Mg^{2+} or Mn^{2+} for its activity; in fact these ions showed some inhibitory effects at the concentrations tested. Similar effects of bivalent cations have also been observed on the rat nuclear PP1 [4]. NaF, a strong inhibitor of Ser/Thr protein phosphatases, totally blocked the activity of the pea enzyme at 5 mM. Molybdate and vanadate, known as strong inhibitors of PTPase, had no obvious effects on the pea PP1 phosphatase activity under test conditions previously shown to inhibit a pea PTPase-like phosphatase [30]. Citrate and tartrate, two inhibitors of acid phosphatase [34], had different effects on the pea phosphatase activity: citrate blocked the phosphatase activity almost totally at a concentration of 5 mM, whereas tartrate showed only slight inhibitory effects at the same concentration (Table 2).

In addition to the ions tested in Table 2, NaCl was also tested. It was found to inhibit phosphatase activity in a concentration-dependent manner. The IC_{50} was 70 mM, and 300 mM almost totally blocked the phosphatase activity (Figure 4), similarly to the effects of KCl on the cardiac sarcoplasmic PP1 [35].

Figure 5 Dephosphorylation of nuclear phosphoproteins by pea nuclear PP1

The nuclear proteins were phosphorylated with endogenous protein kinases. The dephosphorylation reactions were stopped at the time points indicated by adding the SDS sample buffer. The proteins were separated by SDS/PAGE [12% (w/v) gel]. The level of dephosphorylation was judged by the decrease in the intensities of the labelled protein bands in the autoradiogram. In the control experiment (60 min + OA), 1 μ M okadaic acid (OA) was added to the reaction mixture before the reaction was initiated.

Phosphorylation and dephosphorylation of endogenous nuclear protein(s)

The two phosphorylation patterns of nuclear proteins phosphorylated by endogenous kinases by the two different methods described are essentially indistinguishable as assayed by autoradiography after SDS/PAGE. Both methods show that the two major phosphorylated bands have molecular masses of 92 and 43 kDa (Figure 5). When the phosphorylated nuclear proteins were treated by the phosphatase, the labelling intensity of only the 92 kDa protein was decreased in a time-dependent manner, whereas the 43 kDa protein was not affected. The decrease in the labelling intensity of the 92 kDa band was inhibited by okadaic acid (Figure 5), indicating that this was not due to the action of proteases that might have been present in the enzyme preparation. Also, because the nuclear phosphoprotein substrates were collected by precipitation with trichloroacetic acid, it was highly unlikely that their dephosphorylation was due to any endogenous phosphatase activity. Whether other minor phosphoproteins were dephosphorylated by the phosphatase could not be distinguished by this experiment.

DISCUSSION

High levels of PP1 have been reported in the nuclei of animal cells [4–6], where it accounts for most if not all of the phosphorylase *a* phosphatase activity under the conventional assay conditions. Strikingly, it is estimated that 90% of this phosphatase activity is found in the chromatin fraction of animal nuclei [5]. Similar to the case in animal cells is our observation that most of the phosphorylase phosphatase activity in pea nuclei was also associated with the chromatin fraction, and most of this was of the PP1 type. The fact that the pea nuclear phosphatase remained associated with the chromatin fraction after treatment with Triton X-100 argues against its having only a surface association with nuclei that occurred as an artifact during the extraction procedure.

When the phosphatase activity assay was performed in the presence of 2 nM okadaic acid, only 10–20% of the total phosphorylase phosphatase activity was inhibited in both the Triton X-100 fraction and the chromatin extracts, indicating that

at least 80% of the extractable phosphatase activity in pea nuclei was PP1 activity.

The molecular mass of the phosphatase is about 34 kDa as estimated by molecular sieve chromatography, which is similar to that of the animal type 1 catalytic subunit (PP1c), including rat nuclear PP1c [4–6]. The active fractions from the IEF gel migrated to a pI in the range 5.4–5.5, and the extracts from the IEF active fractions contained only one silver-stainable protein band with a molecular mass of approx. 40 kDa on SDS/PAGE (Figure 2), indicating that this is most probably the protein with phosphatase activity. Although a protein of approx. 35 kDa was one of the four major proteins present in the sample fractionated by IEF, it was not detectable in the IEF fractions that contained all the phosphatase activity. To our knowledge, PP1 has not been purified previously from plant cells. Several cDNAs encoding PP1-like enzymes have been isolated from different plant species [20–22,24], and the genes for PP1 apparently exist as a multi-member gene family. Five distinct cDNA clones encoding PP1c were isolated from *Arabidopsis*. The predicted molecular masses of the proteins that they encode range from 35.5 to 36.2 kDa, and the pI values range from 4.94 to 5.82 [22].

Most probably the pea nuclear phosphatase isolated represents the PP1c; in other words it was not associated with any regulatory subunit under our purification conditions. The rat nuclear PP1 was isolated both as monomeric PP1c [4,5] and as a complex of PP1c and endogenous nuclear inhibitor peptides [6] that regulate its activity. Whether such regulatory subunits exist in the plant cell is currently unknown.

The activity of the pea nuclear phosphatase was characterized by using 32 P-labelled phosphorylase *a* as a substrate. This phosphoprotein is conventionally used as a substrate in the characterization of animal PP1 and PP2A and of their specific inhibitors [1]. The specific activity of the nuclear phosphatase preparation after the final column purification step was approx. 4000 nmol/min per mg (Table 1). However, this specific activity was probably substantially underestimated, for it was measured in an assay buffer containing a final concentration of 66 mM NaCl as a result of using buffer D (which included 0.2 M NaCl) to dilute the sample before assay. We found that decreasing the NaCl concentration of the chromatin extracts caused precipitation of some nuclear proteins and the loss of some phosphatase activity, similar to the situation during the purification of rat liver PP1, in which dilution of the NaCl concentration in the crude chromatin extract to below 0.4 M resulted in aggregation of proteins and decreased phosphatase activity [4]. This precipitation response to dilution was marked at earlier stages of purification but less pronounced or absent as the overall protein concentration was decreased in the later stages of purification. None the less, to increase the final yield of the purification protocol, the salt-containing buffer D was used for the dilution of enzyme preparations. Although the presence of NaCl prevents protein precipitation, it also inhibits the phosphatase activity in a concentration-dependent manner with an IC_{50} of about 70 mM (Figure 4). Therefore the specific activities measured during purification in Table 2 represent only approx. 50% of the actual specific activities that would be measured in the absence of NaCl. Thus the specific activity of the final preparation without NaCl would be near 8000 nmol/min per mg. If we allow that the 40 kDa band in the inset of Figure 2 is the PP1c and that it represented approx. 20% of the total protein in the final preparation, the expected specific activity of pure pea nuclear PP1 would be near 40000 nmol/min per mg, very close to that reported for pure mammalian PP1s.

The organic inhibitors tested are especially useful for distinguishing different classes of protein phosphatases. They can

discriminate between different phosphatases even in crude extracts of animal and plant tissues as well as in studies *in vivo* [12,17,19]. The fact that three of the most widely used protein phosphatase inhibitors, okadaic acid, inhibitor 2 and microcystin-LR, produced similar inhibition profiles when tested against the pea nuclear phosphatase to those when they are tested against animal PP1 [5,7] and plant PP1 [11] strongly supports the conclusion that the pea enzyme is a PP1-type phosphatase.

This conclusion is also supported by the assays of ion effects, for Ca^{2+} , Mg^{2+} or Mn^{2+} affect the activity of the pea phosphatase as they do the activity of the rat nuclear PP1 [4]. One inhibitor of acid phosphatase, citrate, also strongly inhibited the pea PP1, whereas another, tartrate, showed little effect on the enzyme activity (Table 2). Citrate is not generally tested against PP1 enzymes, so we are not certain whether this is a unique effect of citrate on the pea PP1 or whether it is a more general inhibitor of PP1-type phosphatases.

At millimolar concentrations, vanadate and molybdate are generally effective inhibitors of phosphatases, as well as some other enzymes such as ATPase. However, even at micromolar concentrations these two ions still strongly inhibit PTPases and so are widely used at these very low levels to identify PTPases diagnostically [36]. For example, micromolar concentrations of vanadate selectively inhibit the dephosphorylation of phospho-Tyr-proteins, whereas the dephosphorylation of phospho-Ser/Thr-proteins is not affected under the same conditions [37], indicating that Ser/Thr protein phosphatases are not sensitive to micromolar levels of these ions. Similarly, micromolar levels of vanadate and molybdate have no effect on the pea nuclear PP1, but as reported by Guo and Roux [30] they almost totally block the activity of a PTPase-like enzyme from pea nuclei [30] and a PTPase-like enzyme isolated from the cytosolic fraction of pea plumules under the same conditions (Y.-L. Guo and S. J. Roux, unpublished work).

The results presented in this report clearly classify the pea protein phosphatase as belonging to the PP1 type, on the basis of the criteria used in the classification of animal protein phosphatases. Its major biochemical and enzymic properties, e.g. its substrate specificity, its inhibitor profile and the effects of ions on its activity, are all very similar to those of well-characterized animal PP1 enzymes [1,2] and to those of partly characterized plant PP1-like enzymes [11,12].

Along with the PP1 protein phosphatase, the protein extracted by 0.35 M NaCl from the pea nuclear chromatin fraction contains at least two protein kinases (a CDPK [28] and a CK-2 [29]) and a PTPase-like phosphatase [30]. Also in this extract are several pea nuclear proteins that can be phosphorylated by the endogenous kinases. Among them, two proteins of 92 and 43 kDa are heavily phosphorylated. The 43 kDa protein is a preferred substrate of pea CDPK [28] and its phosphorylation is stimulated by Ca^{2+} . It is interesting to note that pea PP1 selectively dephosphorylated the 92 kDa band whereas the 43 kDa protein was not affected. Currently we do not know the identities of these proteins, but preliminary results indicate that the 92 kDa band includes a nucleolin-like protein (C.-G. Tong, S. Reichler, J. Balk and S. J. Roux, unpublished work). Nucleolin is a major nucleolar protein that is heavily phosphorylated by CK-2 and can be dephosphorylated by PP1 in animal cells [1].

In summary, the pea nuclear phosphatase resembles animal nuclear PP1 in its biochemical and enzymic properties, in the fact that it occurs in a high level in the nucleus, and in its physical association with chromatin. It is reasonable to speculate that the pea PP1 might also resemble the animal nuclear PP1 functionally and thus play a similar role in the regulation of such nuclear events as cell cycle transitions and transcriptional activation and repression.

We thank Dr. G. Kramer for providing the partly purified PKA, and Dr. Wenfeng Wang for providing the rabbit muscle inhibitor 2. This work was supported by a grant from the National Science Foundation (DCB 9106245) to S. J. R.

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