

# A Cyclophilin-Regulated PP2A-like Protein Phosphatase in Thylakoid Membranes of Plant Chloroplasts<sup>†</sup>

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**ABSTRACT:** Dephosphorylation of central photosynthetic proteins regulates their turnover in plant thylakoid membranes. A membrane protein phosphatase from spinach thylakoids was purified 13000-fold using detergent-engaged FPLC. The purified enzyme exhibited characteristics typical of eukaryotic Ser/Thr phosphatases of the PP2A family in that it was inhibited by okadaic acid ( $IC_{50} = 0.4$  nM) and tautomycin ( $IC_{50} = 25$  nM), irreversibly bound to microcystin–agarose, and recognized by a polyclonal antibody raised against a recombinant catalytic subunit of human PP2A. Furthermore, the anti-PP2A antibody inhibited protein dephosphorylation in isolated thylakoids. The phosphatase copurified with TLP40, a cyclophilin-like peptidyl-prolyl isomerase located in the thylakoid lumen. TLP40 could be released from the phosphatase immobilized on microcystin–agarose by high-salt treatment. Binding of cyclosporin A (CsA) to TLP40 led to thylakoid phosphatase activation, while cyclophilin substrates, prolyl-containing oligopeptides, inhibited protein dephosphorylation. This dephosphorylation could be modulated by CsA or oligopeptides only after the thylakoids had been ruptured to expose the luminal membrane surface where the TLP40 is located. Regulation of the PP2A-like phosphatase at the outer thylakoid surface is likely to operate via reversible binding of TLP40 to the inner membrane surface. This is a first example of transmembrane regulation in which the activity of phosphatase is altered by the binding of a cyclophilin to a site other than the active one. We propose that signaling from TLP40 to the protein phosphatase coordinates dephosphorylation and protein folding, two processes required for protein turnover during the repair of photoinhibited photosystem II reaction centers.

Excess light can induce photoinactivation of plant photosynthetic electron transport and cause oxidative damage to the photosystem II reaction center (1). To minimize damage, plants have evolved a number of specific acclimative and protective mechanisms, one of them being light-induced phosphorylation of chloroplast thylakoid membrane proteins (2–4). The main targets of this phosphorylation are components of the major chlorophyll *a/b*-binding protein complex (LHCII)<sup>1</sup> and of the photosystem II (PSII) core complex (2–

5), including the two reaction center proteins D1 and D2. These proteins are phosphorylated on threonine residues at the outer surface of the thylakoid membrane, and their reversible phosphorylation is thought to determine the partitioning of excitation energy between photosystems I and II (2, 3) and to control the turnover of PSII reaction center subunits (5). The kinases responsible for phosphorylation of thylakoid proteins are intrinsic components of the membrane. Their activities are under redox control (2–7) which involves plastoquinol binding to the reduced cytochrome *bf* complex (8, 9). A family of thylakoid membrane-associated protein kinases (TAKs) which appear to phosphorylate LHCII subunits has recently been identified (10). Thylakoids are also known to contain several protein phosphatases. A number of extrinsic membrane phosphatases (11, 12) and phosphatases from the chloroplast stroma (13, 14) capable of dephosphorylating some thylakoid proteins have been identified and partially characterized. However, the dephosphorylation of thylakoid phosphoproteins was shown to be catalyzed predominantly by phosphatases integral to the

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<sup>1</sup> Abbreviations: CsA, cyclosporin A; DM, *N*-dodecyl  $\beta$ -D-maltoside; LHCII, major light-harvesting chlorophyll *a/b*-protein complex of photosystem II; P<sub>i</sub>, inorganic phosphate; PPIase, peptidyl-prolyl cis–trans isomerase; PSII, photosystem II; TLP40, 40 kDa thylakoid lumen peptidyl-prolyl cis–trans isomerase.

thylakoid membrane (15). These intrinsic membrane phosphatases have thus far escaped identification, and very little is known about their regulation.

Eukaryotic Ser/Thr protein phosphatases are typically classified according to their substrate specificity, requirement for divalent cations, and susceptibility to inhibitors (16–19). By these criteria, two major families have been identified: the  $Mg^{2+}$ -dependent PPM family which includes PP2C and the  $Mg^{2+}$ -independent PPP family which includes subfamilies PP1, PP2A, and PP2B. The enzymatic activities of PPP phosphatases are regulated in a sophisticated manner by a large number of regulatory and targeting subunits which can bind to their catalytic subunits (reviewed in refs 17–20). For the PP2A subfamily, more than 15 distinct regulatory and targeting proteins have been characterized (18, 19). Specifically, the heterodimeric protein phosphatase PP2B (calcineurin) has been shown to be a target of the peptidyl-prolyl cis–trans isomerases (PPIases) designated immunophilins which function as protein folding catalysts (20, 21) and as receptors of immunosuppressive drugs (22, 23). The inhibition of PP2B by immunophilin–drug complexes is recognized to be a key step leading to the arrest of T cell signal transduction and immunosuppression in mammals (22, 23).

The protein phosphatases of plant thylakoid membranes were previously considered to be unrelated to the PPP family, because they were found to be uninhibited by several typical inhibitors of these enzymes in in membrane dephosphorylation experiments (24, 25). In this work, we purified an intrinsic thylakoid membrane phosphatase and showed that it is related to conventional eukaryotic Ser/Thr protein phosphatases. We found the purified phosphatase to be inhibited by okadaic acid and tautomycin in a manner similar to that of PP2A. Furthermore, we found that its activity is regulated by the cyclophilin-like protein TLP40, an immunophilin present within the thylakoid lumen. Additionally, our results provide evidence for cyclophilin-controlled signal transduction in plant chloroplasts which may regulate protein turnover and the repair of photoinhibitory damage to PSII.

## MATERIALS AND METHODS

**Plants and Materials.** Thylakoid membranes were isolated from 6-week-old spinach plants (26) that were grown hydroponically as described previously (27). Chlorophyll and protein contents were determined as described previously (28–30). Okadaic acid, tautomycin, CsA, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, and a rabbit polyclonal antibody against a recombinant catalytic subunit of human PP2A lacking amino acids 15–145 were purchased from Calbiochem. Microcystin–agarose was purchased from Upstate Biotechnology, and arachidic acid, palmitic acid, myristic acid, and DL- $\alpha$ -dipalmitoylphosphatidylcholine were purchased from Sigma.

**Preparation of Phosphopeptides and Phosphatase Activity Assays.** To prepare  $^{32}P$ -labeled phosphopeptides, purified thylakoid membranes (1.5 mg of chlorophyll/mL) were incubated under illumination ( $300 \mu\text{mol of photons m}^{-2} \text{ s}^{-1}$ ) for 30 min in 50 mM Tricine-NaOH (pH 7.8), 100 mM sorbitol, 5 mM  $MgCl_2$ , 10 mM NaF, and 1 mM  $[\gamma\text{-}^{32}P]\text{ATP}$  (0.13 mCi/mL). The reactions were stopped by the addition of an equal volume of ice-cold 50 mM EDTA. The

phosphorylated membranes were then collected by centrifugation, washed seven times with 50 mM Tricine-NaOH (pH 7.8) and 100 mM sorbitol and once with 10 mM  $NH_4HCO_3$ , and finally resuspended at the same concentration in 10 mM  $NH_4HCO_3$ . The membrane suspension was incubated with 7  $\mu\text{g/mL}$  trypsin for 30 min at room temperature to cleave and release the surface-exposed phosphopeptides from the thylakoid membranes. Proteolysis was terminated by the addition of soybean trypsin inhibitor at a concentration of 140  $\mu\text{g/mL}$ . After centrifugation, the supernatant containing the radiolabeled phosphopeptides was collected, heated at 90 °C for 10 min, and dried in aliquots in a SpeedVac centrifuge.

To assay protein samples for phosphatase activity using the phosphopeptides, 5  $\mu\text{L}$  of  $^{32}P$ -labeled phosphopeptides dissolved at a concentration of 10–20  $\mu\text{M}$  in 50 mM Tricine-NaOH (pH 7.8) and 5 mM  $MgCl_2$  was incubated with an equal volume of protein sample for 10–60 min at room temperature. Following the addition of 0.2 mL of 0.5 M  $H_2SO_4$  and 1 mM  $KH_2PO_4$  to stop the reaction, the released inorganic phosphate was converted to a phospho–molybdate complex by the addition of 80  $\mu\text{L}$  of 5% (w/v) ammonium molybdate and extracted with 0.4 mL of isobutanol/heptane (1:1, v/v). The phosphate in the organic phase was separated from the phosphopeptides in the water phase and quantified in a scintillation counter according to the method of Cherenkov.

To assay protein samples for phosphatase activity using intact thylakoids, the membranes phosphorylated by  $[\gamma\text{-}^{32}P]\text{ATP}$  as described above were washed once with ice-cold 50 mM EDTA and three times with 50 mM Tricine (pH 7.8) and 100 mM sorbitol and incubated at 55 °C for 5 min to inactivate the endogenous phosphatase activities. Equal volumes of the membrane preparation and the protein fraction of interest were incubated at room temperature for 45 min. Following addition of electrophoresis sample buffer, the samples were heated at 70 °C for 4 min and subjected to SDS–PAGE (31) on 15% acrylamide slab gels. Following electrophoresis, the gels were stained, dried, and autoradiographed. The relative phosphorylation level of distinct proteins was quantified using laser densitometry of the X-ray films, with the software package Image Quant from Molecular Dynamics.

To assay endogenous phosphatase activity in thylakoids, the membranes phosphorylated using  $[\gamma\text{-}^{32}P]\text{ATP}$  were washed to remove excess low-molecular weight  $^{32}P$  as described above and resuspended in 50 mM Tricine (pH 7.8), 100 mM sorbitol, 5 mM  $MgCl_2$ , and 2 mM DTT. Following incubation for 15–120 min at room temperature, the samples were analyzed by SDS–PAGE and autoradiography as described above.

**Purification of a Protein Phosphatase from Thylakoid Membranes.** Thylakoids isolated from 300 g of spinach leaves were washed twice with 2 M NaBr in 20 mM Tricine-NaOH (pH 7.8), 0.4 M sorbitol, and 2 mM DTT to remove extrinsic proteins (32), solubilized with 1% Triton X-100 for 30 min at 4 °C, and centrifuged for 1 h at 100000g. The resulting supernatant was loaded onto a 15 mL Sepharose Q column which had been equilibrated with 0.5% Triton X-100, 20 mM Tricine-NaOH (pH 7.8), and 2 mM DTT. The column was then washed with 75 mL of the same buffer supplemented with 0.1 M NaCl, after which a phosphatase-

containing fraction (25 mL) was eluted in buffer containing 0.3 M NaCl. The sample was concentrated and desalted using an Amicon 10 kDa cutoff membrane and subjected to two consecutive FPLC runs on a 1 mL Resource Q column. Fractions of the first run were eluted using 30 mL of a 0 to 0.5 M NaCl linear gradient in 20 mM Tricine-NaOH (pH 7.8), 2 mM DTT, and 2 mM DM. Fractions containing phosphatase activity were reloaded onto the Resource Q column and eluted with the same buffer as before except that 8 mM CHAPS was used instead of DM. The fractions which contained phosphatase activity were pooled and concentrated to 0.1 mL and subjected to FPLC on a Superose 12 (HR 10/30) column equilibrated with 50 mM imidazole-HCl (pH 6.0), 2 mM DM, and 2 mM DTT using a flow rate 0.3 mL/min. For affinity chromatography, phosphatase-containing fractions from the Superose 12 column were loaded onto a 0.5 mL microcystin-agarose column with a flow rate of 0.02 mL/min. The column was then washed with (i) 50 mM triethanolamine-HCl (pH 7.5), 0.1 mM DM, 1 mM MnCl<sub>2</sub>, and 2 mM DTT, (ii) the same buffer with a 0 to 1.0 M NaCl gradient at a flow rate of 0.15 mL/min, and (iii) the same buffer containing 3 M NaSCN.

**Limited Proteolysis of Thylakoid Membranes.** To release the catalytic domain of the protein phosphatase from thylakoid membranes, NaBr-washed (see above) membranes (1.5 mg of chlorophyll) in 1 mL of 50 mM Tricine-NaOH (pH 7.8), 5 mM MgCl<sub>2</sub>, and 2 mM DTT were incubated with 3  $\mu$ g of trypsin at 22 °C. Proteolysis was terminated by addition of 1 mM phenylmethanesulfonyl fluoride. The released polypeptides were separated from the membranes by centrifugation for 30 min at 100000g.

**Peptidyl-Prolyl Isomerase Assay.** The peptide substrate *N*-succinimidyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was used to assay PPIase activity in a two-step, coupled reaction involving chymotrypsin (33). The reaction was started by addition of peptide substrate up to a concentration of 50–70  $\mu$ M in the reaction mixture containing 30–50  $\mu$ M chymotrypsin and 0.05  $\mu$ M TLP40 in a final volume of 1 mL of Tricine-NaOH (pH 7.8) pre-equilibrated at 10 °C in the spectrophotometer cuvette. The absorbance at 390 nm was recorded using a Shimadzu UV-3000 spectrophotometer. Inhibition by CsA was assayed after preincubation of the protein samples or thylakoid membranes with 1 nM to 1 mM CsA in the presence of 2–10 mM DM for 15–60 min. When the PPIase activity of intact or ruptured thylakoids was assayed, the chlorophyll concentration was 20  $\mu$ g/mL in both sample and reference cuvettes.

**Thylakoid Membrane Subfractionation.** Thylakoid membranes were fragmented by a Yeda press and subfractionated by centrifugation and aqueous polymer two-phase partitioning (34). The fractions enriched in right-side-out, inside-out, appressed, and stroma-exposed thylakoid membranes as well as a supernatant fraction containing soluble luminal proteins were analyzed by SDS-PAGE and Western blotting with specified antibodies. Gel loading was on an equal chlorophyll basis. The proportion of each protein in each subfraction was determined using ECL (Amersham) fluorography.

## RESULTS

**Assay for Thylakoid Protein Phosphatase Activity.** To elucidate the enzymology of protein dephosphorylation in

thylakoid membranes, we sought to purify and classify any membrane-bound phosphatase enzymes. So far, intrinsic thylakoid phosphatase activity has only been assayed with phosphorylated thylakoids, mimicking synthetic phosphopeptides (35, 36), and phosphopeptides released from thylakoids by chymotrypsin (12). To prepare phosphopeptide substrates, we phosphorylated spinach thylakoids using [ $\gamma$ -<sup>32</sup>P]ATP and the endogenous light-activated protein kinase. Thereafter, the membranes were extensively washed to remove nonreacted [ $\gamma$ -<sup>32</sup>P]ATP, and the phosphopeptides were released from the membranes by trypsin digestion (Figure 1A). Incubation of thylakoid membranes, following pretreatment with 2 M NaBr (32) to remove extrinsic phosphatases, with these phosphopeptides resulted in the time-dependent release of labeled phosphate (Figure 1B). Dephosphorylation of the phosphopeptides was completely inhibited by NaF, a known inhibitor of thylakoid protein dephosphorylation (12, 15). Evidence that the tryptic phosphopeptides do serve as substrates for the endogenous thylakoid protein phosphatases comes from the observation that excess phosphopeptides inhibited the dephosphorylation of phosphoproteins within intact thylakoid membranes (Figure 1C).

**Purification of a Thylakoid Membrane Protein Phosphatase.** To purify membrane phosphatases, spinach thylakoids were depleted of extrinsic proteins and solubilized with the nonionic detergent Triton X-100. This lysate was subjected to anion-exchange chromatography on a Sepharose Q column. Fractions with phosphopeptide hydrolyzing activity were subjected to further purification on two successive Resource Q columns. The sample and elution buffers included DM for the first Resource Q column and CHAPS for the second (Figure 2A,B). Combined, these chromatographic separations resulted in a greater than 800-fold purification of the phosphatase (Table 1). This fraction contained a dominant 40 kDa protein and five to seven less abundant polypeptides with lower molecular masses (Figure 2D, lane 6). This 40 kDa protein was microsequenced, cloned, and characterized as a novel complex cyclophilin-like PPIase, which we named TLP40 (37).

The final purification step, Superose 12 chromatography, resolved a phosphatase activity from the majority of TLP40 (Figure 2C). The TLP40 fraction appeared to contain only the 40 kDa protein (Figure 2D, lane 7a), while the fraction containing the 13000-fold-purified phosphatase (Table 1) appeared to consist of TLP40 and three to four polypeptides with lower molecular masses (Figure 2D, lane 7b). A 39 kDa protein in fraction 7b was recognized by an antibody raised against the catalytic subunit of the protein phosphatase PP2A (see below). Catalytic subunits of PP2A phosphatases always appear to be bound by at least two regulatory factors (17–20). As demonstrated below, TLP40 acts as a regulatory subunit of the phosphatase. Whether any of the other polypeptides present in fraction 7b are components of the TLP40-phosphatase complex is currently unknown. Efficient separation of the phosphatase complex from the TLP40 monomer was achieved because of its long retention time on the Superose 12 column, which could be due to an affinity of the complex for the matrix.

**Characterization of the Highly Purified Protein Phosphatase.** To test the purified phosphatase for its ability to dephosphorylate thylakoid phosphoproteins, the enzyme was



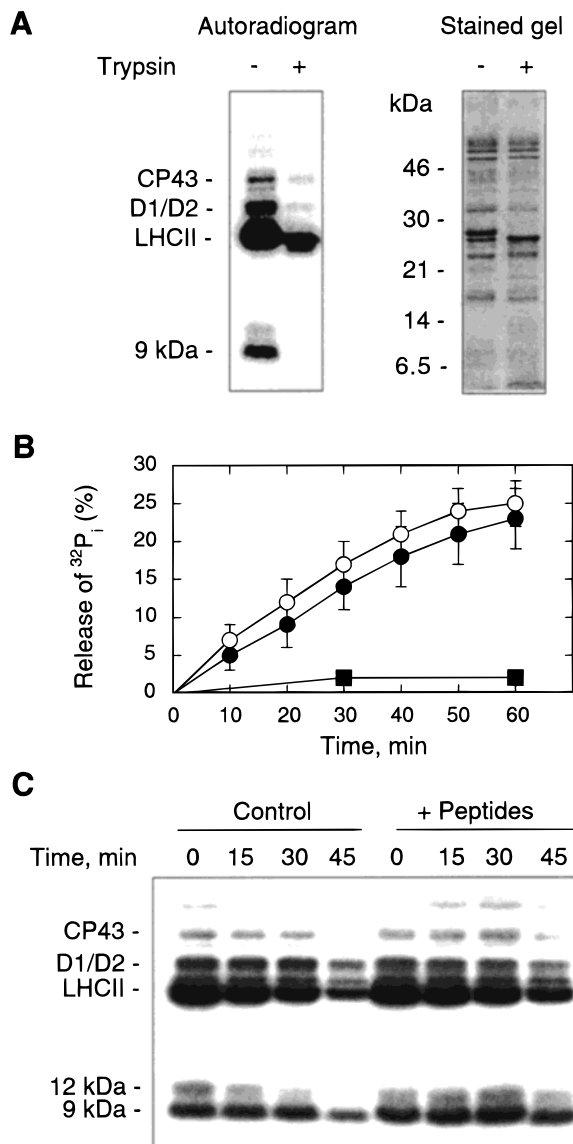


FIGURE 1: Using  $^{32}\text{P}$ -labeled phosphopeptides released from thylakoid membranes by trypsin cleavage to assay protein phosphatase activity. (A) Autoradiogram and Coomassie-stained gel showing the profiles of thylakoid membrane proteins before and after treating  $^{32}\text{P}$ -phosphorylated thylakoids with trypsin. (B) Graph depicting the release of radioactive phosphate from the  $^{32}\text{P}$ -labeled phosphopeptides over time. The phosphatase was supplied as intact thylakoid membranes (○) or as Triton X-100-solubilized membranes either in the absence (●) or in the presence of 10 mM NaF (■). (C) Competition between tryptic phosphopeptides and thylakoid phosphoproteins for dephosphorylation by the protein phosphatase in intact membranes. The autoradiogram shows the dephosphorylation of  $^{32}\text{P}$ -labeled thylakoids over time in the absence (control) and presence of 30  $\mu\text{M}$  tryptic peptides.

incubated with phosphorylated thylakoid membranes in which the endogenous phosphatase activity had been inactivated by heat treatment. The isolated phosphatase proved to be able to dephosphorylate proteins in these thylakoid membranes (Figure 3A). Notably, the purified enzyme very rapidly dephosphorylated the CP43, D1, and D2 proteins of PSII (Figure 3B). Thus, we presume that the isolated membrane protein phosphatase is the enzyme responsible for PSII dephosphorylation. The previously identified stromal phosphatases were shown to be most active toward phosphoproteins of the LHCII complex (14, 35). One trivial but possible explanation for the slow dephosphorylation of

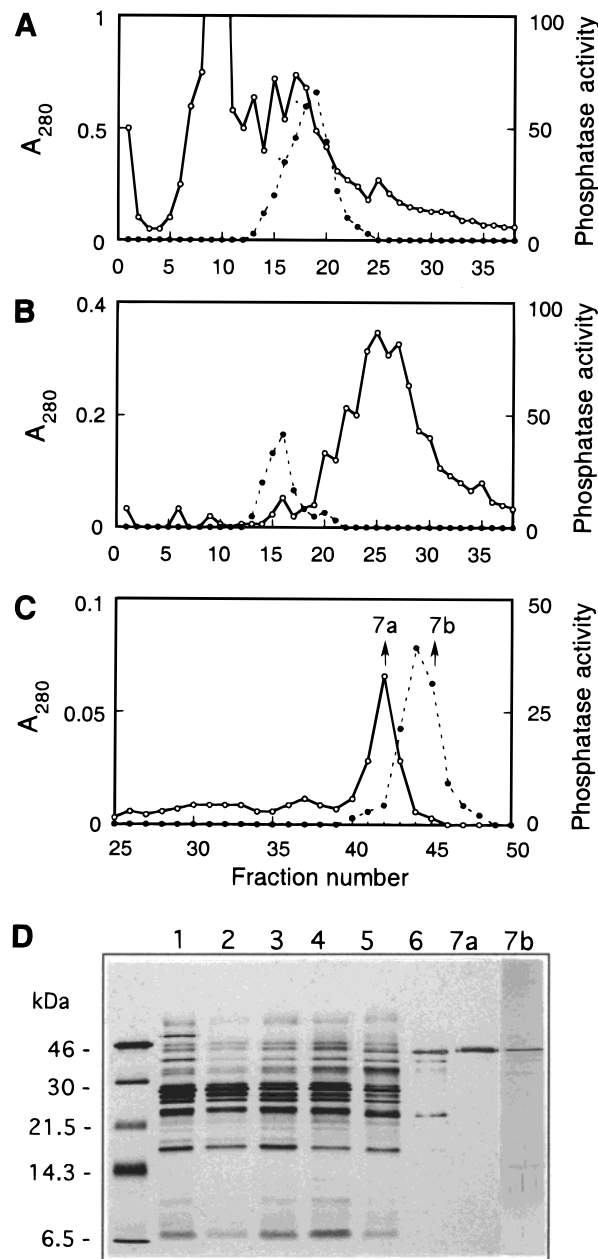


FIGURE 2: Chromatographic purification of a thylakoid membrane protein phosphatase. Graphs A–C depict the total amount of protein [absorbance reading at 280 nm (A<sub>280</sub>, continuous line)] and phosphatase activity (picomoles of  $^{32}\text{P}$  released from the phosphopeptides in 1 min, dashed line) collected in each column fraction during three successive column purification steps. (A) Fractions were eluted from the first Resource Q column with a linear 0 to 0.5 M NaCl gradient in a buffer containing 2 mM DM (fractions 6–35). (B) Fractions were eluted from the second Resource Q column with a linear 0 to 0.5 M NaCl gradient in a buffer containing 8 mM CHAPS (fractions 1–30). (C) Fractions were eluted from a Superose 12 column in a buffer containing 2 mM DM. Fractions 42 and 45 correspond to rows 7a and 7b of Table 1, respectively. (D) Silver-stained gel showing the protein profile of the pooled phosphatase-containing fractions following each chromatographic purification step, as numbered in Table 1.

LHCII and the 12 kDa phosphopeptide by the purified enzyme (Figure 3) is that the heat treatment used to inactivate the endogenous thylakoid phosphatase caused structural changes in these phosphoprotein substrates.

The enzyme activity of the isolated phosphatase was maximal between pH 7.0 and 8.0 when assayed with the

Table 1: Purification of an Intrinsic Protein Phosphatase from Spinach Thylakoid Membranes<sup>a</sup>

purification step	total protein (mg)	total activity (pmol min <sup>-1</sup> )	specific activity (pmol min <sup>-1</sup> mg <sup>-1</sup> )	yield (%)	purification (-fold)
(1) thylakoids	790	2880	3.6		
(2) NaBr-washed membranes	720	1640	2.3	100	
(3) solubilized membranes	610	886	1.5	54	1
(4) Sepharose Q column	42	378	9.0	23	6
(5) Resource Q (I) column	4.5	186	41	11	27
(6) Resource Q (II) column	0.14	178	1270	11	847
(7) Superose 12 column					
(a) fraction 42 <sup>b</sup>	0.04	—	—	—	—
(b) fraction 45	0.002	39	19500	2.4	13000

<sup>a</sup> The protein profile following each purification step is shown in Figure 2D. <sup>b</sup> Contains apparently pure 40 kDa protein (TLP40).

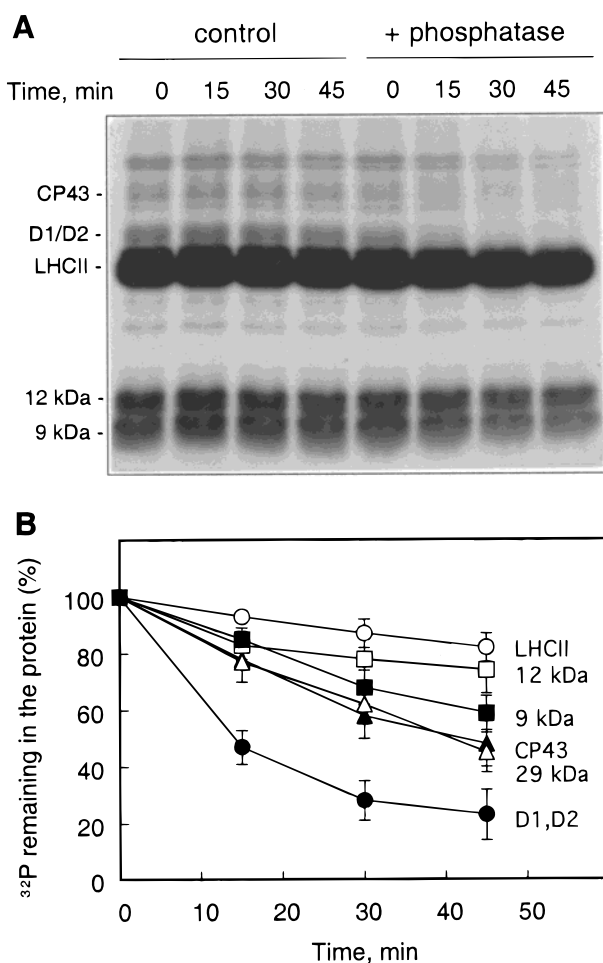


FIGURE 3: Dephosphorylation of thylakoid phosphoproteins by the purified thylakoid membrane protein phosphatase. Autoradiogram of a SDS-PAGE gel (A) and graph (B) showing the dephosphorylation of particular <sup>32</sup>P-labeled thylakoid phosphoproteins over time in the absence (control, panel A only) or presence of the purified phosphatase. The endogenous phosphatase activity in the labeled thylakoid membranes had been inactivated by heat treatment prior to conducting the experiments. The main thylakoid phosphoproteins and their positions are indicated on the left side of the autoradiogram. Each band on the autoradiogram was counted using a densitometer to quantify the level of phosphorylation of each protein. Different exposure times were used to ensure that each phosphoprotein was analyzed in the linear range. Additionally, the values depicted in panel B represent the mean of four experiments.

phosphopeptides (data not shown). Different compounds known to influence catalysis by various phosphatases were monitored for their ability to modulate the activity of the isolated enzyme (Table 2). The phosphatase activity was not

Table 2: Effect of Various Reagents on the Activity of the Purified Membrane Protein Phosphatase

reagent	relative activity (%)
—	100
5 mM MgCl <sub>2</sub>	115 ± 10
1 mM MnCl <sub>2</sub>	110 ± 10
1 mM CaCl <sub>2</sub>	100 ± 10
1 mM NiCl <sub>2</sub>	110 ± 10
1 mM CoCl <sub>2</sub>	55 ± 10
0.3 mM Zn(CH <sub>3</sub> CO <sub>2</sub> ) <sub>2</sub>	0
1 mM CuCl <sub>2</sub>	30 ± 10
5 mM EDTA	90 ± 15
5 mM EGTA	100 ± 10
10 mM NaF	15 ± 5
0.5 mM Na <sub>3</sub> VO <sub>4</sub>	100 ± 10
1 mM Na <sub>2</sub> MoO <sub>4</sub>	95 ± 10
1 mM Na <sub>2</sub> HPO <sub>4</sub>	55 ± 10
15 mM Na <sub>2</sub> HPO <sub>4</sub>	0
5 mM Na <sub>2</sub> H <sub>2</sub> P <sub>2</sub> O <sub>7</sub>	5 ± 5
5 mM ATP	5 ± 5
5 mM MgATP	15 ± 10
1 mM arachidic acid	100 ± 15
1 mM palmitic acid	110 ± 15
1 mM myristic acid	100 ± 15
1 mM dipalmitoylphosphatidylcholine	120 ± 15

significantly inhibited by EDTA or EGTA, nor was it activated by MgCl<sub>2</sub>, MnCl<sub>2</sub>, or CaCl<sub>2</sub>. However, magnesium cations are required for protein dephosphorylation in thylakoids (2, 38); therefore, they may be important for effective presentation of substrates to the phosphatase when in its natural membrane setting but not after it has been solubilized. None of the fatty acids that were tested affected the phosphatase activity. However, millimolar concentrations of Zn(II), fluoride, orthophosphate, pyrophosphate, and ATP caused almost complete inhibition of the purified enzyme (Table 2).

Most strikingly, the highly purified enzyme was inhibited by okadaic acid (IC<sub>50</sub> = 0.4 nM) and tautomycin (IC<sub>50</sub> = 25 nM), two specific inhibitors of eukaryotic Ser/Thr protein phosphatases of the PPP family (Figure 4). These inhibition characteristics are indicative of the PP2A and the recently discovered PP2A-related subfamilies (PP4–PP6) of protein phosphatases (16, 18, 19). Consistent with previous studies (24, 25), however, neither okadaic acid nor tautomycin inhibited protein dephosphorylation in intact thylakoid membranes (Figure 4). Notably, the susceptibility of the phosphatase to these inhibitors increased with each step of protein purification. For example, 0.1 μM okadaic acid resulted in approximately 50 and 95% inhibition of phosphatase activity after the first and second FPLC purification steps, respectively (indicated with a square and a diamond

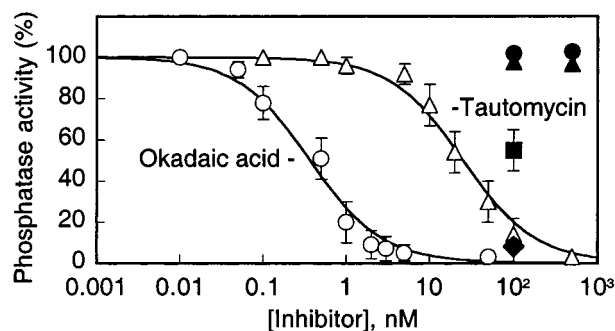


FIGURE 4: Inhibition of the purified protein phosphatase by okadaic acid (○) and tautomycin (△) using radioactive phosphopeptides as substrates. Black circles and triangles represent the effect of okadaic acid and tautomycin, respectively, on endogenous protein dephosphorylation in intact <sup>32</sup>P-labeled spinach thylakoids. The influence of okadaic acid on the phosphatase activity after the Sepharose Q (lane 4 in Figure 2D and Table 1) and Resource Q (lane 5 in Figure 2D and Table 1) chromatography is given by a square and a diamond, respectively.

in Figure 4). The reason the PPP inhibitors did not affect dephosphorylation in intact thylakoid membranes is currently unknown.

**Identification of the Phosphatase Catalytic Subunit.** The catalytic domains of eukaryotic phosphatases of the PPP gene family are highly conserved (24). Therefore, a polyclonal antibody generated against a recombinant human PP2A catalytic subunit was used to identify the catalytic subunit of the thylakoid membrane protein phosphatase. Due to the low abundance of the phosphatase in thylakoid membranes, no protein was detected by Western analysis with this antibody (Figure 5A, lanes T1 and T2). However, it did detect one or two proteins of 37–41 kDa in the concentrated, highly purified phosphatase fractions (Figure 5A, lanes P1 and P2). The banding pattern differed in the two preparations, and it is not clear whether there are two independent PP2A-like catalytic subunits or if one protein becomes post-translationally modified. The antibody against a recombinant human PP2A inhibited endogenous thylakoid protein dephosphorylation in the assay with <sup>32</sup>P-labeled membranes (Figure 5B). The dephosphorylation rates of all thylakoid phosphoproteins in the presence of the anti-PP2A were on average twice as slow as that in the presence of IgG from normal rabbit serum (mean of four experiments). These data suggest that the thylakoid membrane phosphatase is similar to other PP2A enzymes.

PP2A type phosphatases are heterotrimers in which the catalytic subunit of ~36 kDa associates with two regulatory or targeting subunits (18, 19). To examine the possibility that the thylakoid phosphatase consists of a surface-exposed hydrophilic domain with enzymatic activity and a hydrophobic domain which anchors it to the membrane, we performed limited proteolysis of thylakoids using low concentrations of trypsin, chymotrypsin, thermolysine, or endopeptidase Lys-C. The polypeptides released from the membranes with trypsin did exhibit phosphatase activity, although it was decreased due to the protease action (Figure 6A). No remaining activity was detected in the membranes following trypsin treatment. FPLC enrichment of the truncated phosphatase yielded a 34 kDa polypeptide which could be detected with the anti-human PP2A antibody (Figure 6B). Thus, the thylakoid membrane phosphatase appears to consist

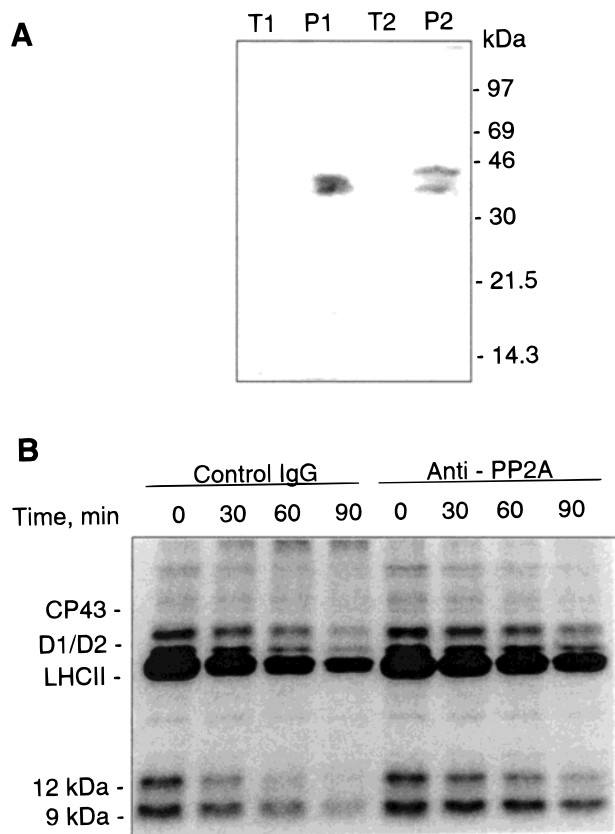


FIGURE 5: Identification of the catalytic subunit of a PP2A-like protein phosphatase from thylakoid membranes. (A) Western blot with a polyclonal antibody generated against the recombinant human PP2A catalytic subunit. Lanes T1 and T2 contained 20 μg of total thylakoid protein. Lanes P1 and P2 contained approximately 0.2 μg of 10000–14000-fold-purified phosphatase from two different preparations. (B) Assay of thylakoid protein dephosphorylation by endogenous phosphatase activity in <sup>32</sup>P-labeled thylakoids in the presence of rabbit IgG from normal serum and the polyclonal antibody generated against the recombinant human PP2A catalytic subunit (autoradiogram). Both antibodies were added to give a final concentration of 12 μg of protein/mL.

of a 34 kDa catalytic domain exposed to the stroma face, a small membrane anchoring domain of 3–7 kDa which could span the membrane once, and possibly a small luminal extension.

**Association of the PP2A-like Phosphatase with TLP40.** PP2A and PP1 type phosphatases are specifically inhibited by microcystin because it becomes covalently bound to the conserved cysteine residue in the active site of these phosphatases (39, 40). Immobilized microcystin has successfully been applied to bind these phosphatases. The catalytic subunits of PP1 phosphatases can be released from microcystin–Sepharose using 3 M NaSCN, but PP2A catalytic subunits cannot (41). Nevertheless, immobilized microcystin can be used to identify PP2A regulatory subunits (42). In an attempt to purify the PP2A-like thylakoid phosphatase more completely and to obtain additional information about its enzymatic properties, FPLC-purified phosphatase fractions were loaded onto a microcystin–agarose column. The enzyme was not eluted from the column with 1 M NaCl, 3 M NaSCN, or 6 M guanidinium chloride. Its binding was irreversible, thus linking the thylakoid phosphatase to the PP2A family. However, TLP40 was released in the eluate of the microcystin–agarose column



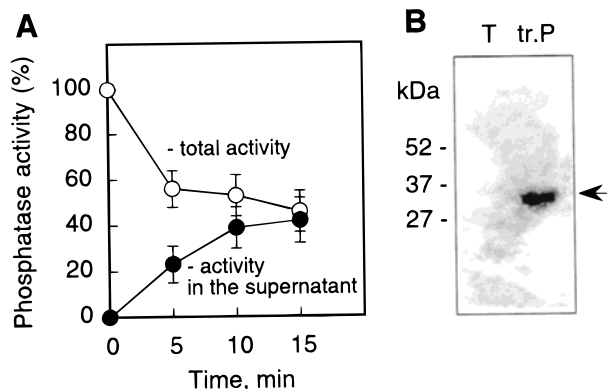


FIGURE 6: Identification of the catalytic domain of the PP2A-like phosphatase after the limited trypsinization of the thylakoid membranes. (A) Time dependence of the phosphatase activity in a suspension of thylakoid membranes incubated with trypsin (total activity) and in the supernatant after centrifugation. (B) Identification of the trypsin-released catalytic domain of the PP2A-like phosphatase by Western analysis with the polyclonal antibody against the recombinant human PP2A catalytic subunit. Lane T contained 20  $\mu$ g of thylakoid protein. Lane tr.P contained the fraction containing the truncated phosphatase which was partially purified and concentrated.

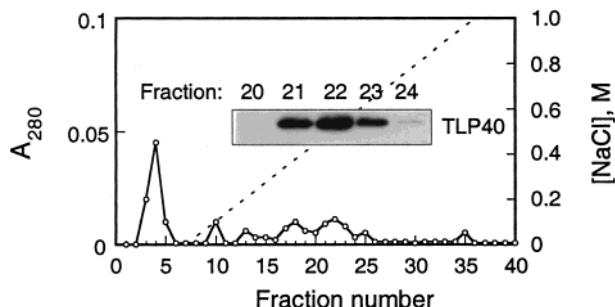


FIGURE 7: Identification of TLP40 in fractions eluted from microcytin-agarose bound with the highly purified thylakoid phosphatase. The protein elution profile ( $A_{280}$ , absorbance at 280 nm, continuous line) and the NaCl concentration of each eluate fraction (dashed line) are shown. The inset shows the content of TLP40 in fractions 20–24 as determined by Western analysis.

when washed with the buffer containing 0.4 M NaCl (Figure 7). This finding, in addition to their obstinate copurification (Figure 2) and their co-immunoprecipitation (37), provided compelling evidence for a direct physical interaction between TLP40 and the thylakoid phosphatase.

**Potential Phosphatase-Binding Sites of TLP40.** TLP40 is the only complex cyclophilin known in plants (37). It is composed of two domains: a catalytic cyclophilin-like C-terminal domain and an N-terminal domain containing leucine zipper consensus sequences (43, 44) with Leu or Ile at every seventh position (Figure 8). The sequence upstream of the first leucine zipper element is 57% identical to the two loops of regulatory subunit A responsible for binding the catalytic subunit of PP2A (45). The sequence downstream of the leucine zipper is 66% similar to the PP2A binding epitope in casein kinase (46) and 100% similar to the PP2B binding site of FKBP12 (47) (Figure 8). Since the interactions between the catalytic subunits of PPP enzymes and their regulatory proteins occur via small binding domains in the regulatory proteins (45–49) and because the plant TLP40 includes regions of amino acid sequence similar to these binding domains, these regions are likely to be the elements in TLP40 which interact with the thylakoid protein phosphatase.

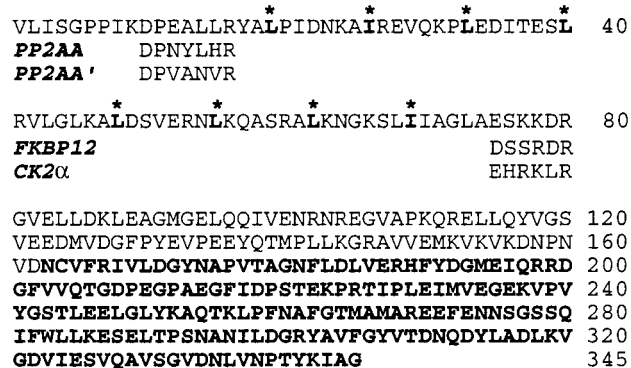


FIGURE 8: Analysis of the deduced amino acid sequence of mature TLP40 (EMBL Data Bank accession number Y12071). The C-terminal sequence representing the cyclophilin-like domain is bold. The leucine and isoleucine residues of the potential leucine zipper in the N-terminus are bold and marked with asterisks. The proposed phosphatase-binding sites, flanking the leucine zipper, are aligned with the well-characterized phosphatase-binding epitopes. *PP2AA* and *PP2AA'* represent residues 492–498 and 531–537, respectively, in the regulatory A subunit of PP2A (45); *FKBP12* designates the PP2B-binding site in FKBP12, residues 37–42 (47), and *CK2 $\alpha$*  is a PP2A-binding motif in casein kinase 2 $\alpha$ , residues 167–172 (46).

**Regulation of Thylakoid Protein Phosphatase by the TLP40 Cyclophilin.** The binding of the immunosuppressive drug CsA to mammalian cyclophilins inhibits PP2B type phosphatase activities (22, 23). To determine if there is functional significance associated with the interaction between TLP40 and the thylakoid protein phosphatase, the effects of adding CsA were investigated. DM was included to rupture the thylakoid membranes, to expose TLP40 located in the lumen, and to increase the solubility of CsA. TLP40 is not sensitive to CsA at concentrations up to 10  $\mu$ M (37), the approximate limit of CsA solubility in water. Intact thylakoids did not exhibit PPIase activity (Figure 9A, trace 1), but following the addition of DM which caused membrane rupture, the luminal TLP40 gained access to the peptide substrate and quickly caused the cis–trans isomerization of the proline residue (Figure 9A, trace 2). The addition of CsA to the ruptured thylakoids inhibited the PPIase activity at sub-millimolar concentrations (Figure 9A, traces 3 and 4). The PPIase activity of purified TLP40 (Figure 2D, lane 7a) assayed in the presence of DM was inhibited by CsA in the same concentration range with an  $IC_{50}$  of 0.18 mM (Figure 9B). The effect of CsA on phosphatase activity in ruptured,  $^{32}$ P-phosphorylated thylakoid membranes was investigated. The rate of thylakoid protein dephosphorylation was significantly increased upon formation of the CsA–TLP40 complex, and all thylakoid phosphoproteins were affected (Figure 9C). As expected, CsA did not affect phosphatase activity when added to intact thylakoid membranes.

Previous studies (23, 50) revealed that CsA bound to human cyclophilin undergoes cis–trans amid isomerization and blocks the cyclophilin active site. To determine if prolyl-containing peptides can also modulate the thylakoid phosphatase activity, we used the conventional PPIase substrate *N*-succinimidyl-Ala-Ala-Pro-Phe-*p*-NA and three other prolyl-containing peptides in the protein dephosphorylation assay with ruptured  $^{32}$ P-phosphorylated thylakoid membranes. The prolyl-containing peptides caused significant inhibition of membrane phosphoprotein dephosphorylation in the ruptured thylakoids (Figure 10). Notably, control peptides without

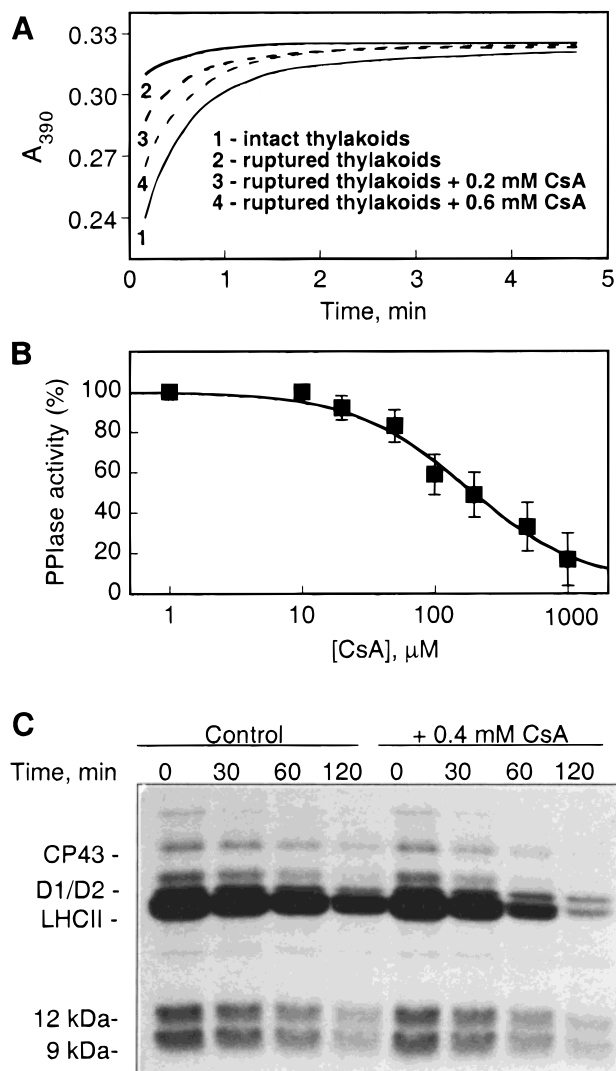


FIGURE 9: Inhibition of the PPIase activity of TLP40 and activation of thylakoid protein dephosphorylation by high concentrations of CsA. (A) Appearance of the PPIase activity after disruption of the thylakoid membranes by DM and inhibition of this activity by CsA. Curve 1 represents an uncatalyzed prolyl *cis*–*trans* isomerization of the substrate peptide *N*-succinimidyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in the presence of intact thylakoids. Curve 2 illustrates almost complete isomerization of the peptide within the mixing dead time in the presence of ruptured thylakoids due to the accessibility of the luminal TLP40. Curves 3 and 4 show inhibition of the PPIase activity of the ruptured thylakoids by 0.2 and 0.6 mM CsA, respectively. (B) Inhibition of the purified TLP40 by different concentrations of CsA in the presence of 5 mM DM. (C) Autoradiogram showing the stimulation of endogenous protein phosphatase activity by 0.4 mM CsA in  $^{32}\text{P}$ -labeled thylakoid membranes ruptured with DM.

prolyl residues did not affect dephosphorylation (Figure 10). Moreover, the prolyl-containing peptides did not influence the phosphatase activity in intact thylakoids (Figure 10), indicating that the peptides do not inhibit phosphatase activity by binding to the enzyme's active site and supporting a role of the luminal TLP40 in regulating phosphatase activity.

**Association of TLP40 with the Inner Thylakoid Membrane Surface.** The connection between the membrane phosphatase with its active site exposed outside of the thylakoid and TLP40 residing inside the lumen prompted us to conduct subfractional analyses to elucidate its topographical basis. Thus, thylakoid membranes were disrupted with a Yeda

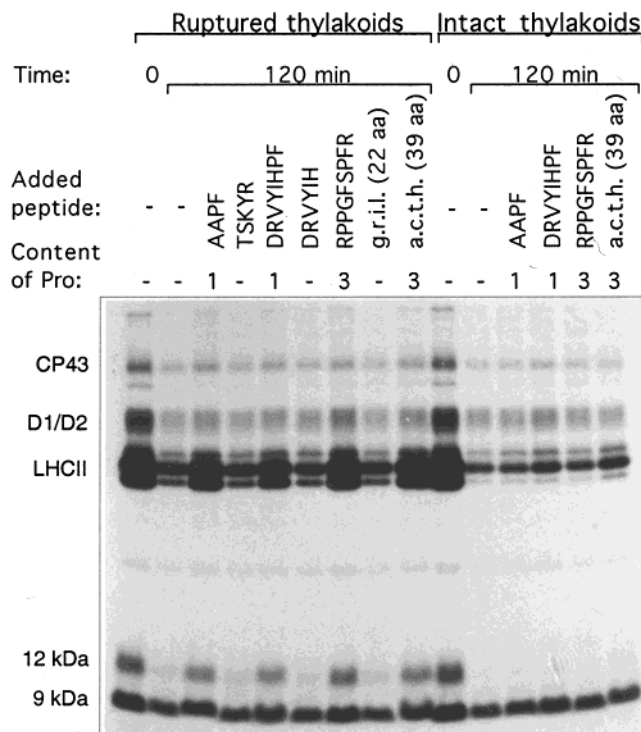


FIGURE 10: Inhibition of thylakoid protein dephosphorylation by prolyl-containing peptides. The autoradiogram demonstrates the relative levels of radiolabel in proteins of  $^{32}\text{P}$ -labeled thylakoids, intact or ruptured with DM, before and after incubation for 120 min with the indicated oligopeptides at a concentration of 4 mM. The single-letter amino acid code is used for the short peptides (AAPF was purchased from Calbiochem; the others were from Bachem). a.c.t.h. (39 amino acids) stands for adrenocorticotrophic hormone (Sigma) and g.r.i.l. (22 amino acids) for the peptide DRVYVAIVHSRRSSSKRVSRNAL-amide. The presence and number of the proline residues in the various oligopeptides are indicated.

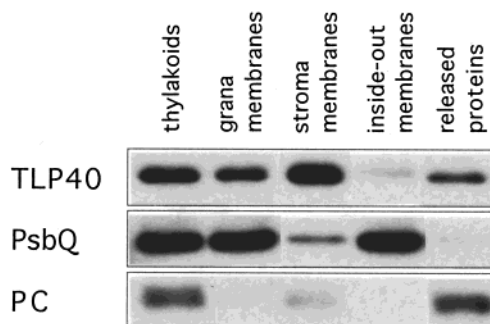


FIGURE 11: Distribution of TLP40, the 23 kDa protein of PSII (PsbQ), and plastocyanin (PC) in subfractions of thylakoid membranes and in the thylakoid lumen. Thylakoid membranes were fragmented by Yeda press and subfractionated by centrifugation combined with aqueous polymer two-phase partitioning. The proteins were detected by Western blot analyses.

press, and the resulting vesicles were subfractionated by differential centrifugation and aqueous polymer two-phase partitioning (34). As judged by immunological analyses, the fragmentation released part of the TLP40 into the aqueous medium, as expected on the basis of its localization to the thylakoid lumen (37). However, a substantial fraction of TLP40 was found to be associated with the membrane subfractions, particularly with the stroma vesicles (Figure 11). Moreover, a minor fraction of the TLP40 was associated with the inside-out vesicles, an indication that it binds to the inner thylakoid surface. The relative binding of TLP40



was compared to that of two other luminal proteins. A smaller portion of TLP40 was bound to the inside-out vesicles than of the 23 kDa PSII protein PsbQ but a larger portion than that of plastocyanin (Figure 11). Thus, it is possible that the binding and release of TLP40 to the inner thylakoid surface is dynamic and that it serves to regulate the activity of the membrane protein phosphatase.

## DISCUSSION

The characterization of the thylakoid membrane protein phosphatase as a PP2A-like enzyme and the finding that cyclophilin TLP40 functions as a phosphatase regulatory component provide insights into the control of the status of thylakoid protein phosphorylation. A number of soluble phosphatases capable of dephosphorylating thylakoid phosphoproteins have previously been isolated (13, 14, 51). However, the membrane-bound intrinsic protein phosphatases have resisted isolation, although they were originally discovered in 1980 (15). One major reason is the difficulty in purifying low-abundance membrane proteins in a functional form. Our protocol involved various detergents at sequential FPLC steps and resulted in a 13000-fold purification of the membrane phosphatase.

The purified intrinsic thylakoid phosphatase was found to possess the characteristics of a conventional eukaryotic Ser/Thr protein phosphatase. The enzyme is (i) specifically inactivated by okadaic acid and tautomycin, (ii) irreversibly bound to immobilized microcystin, and (iii) recognized by a polyclonal antibody raised against a recombinant catalytic subunit of human PP2A. The  $IC_{50}$  values of okadaic acid and tautomycin suggest that the phosphatase closely resembles PP2A (16, 19, 52). However, these inhibitors also similarly inactivate the recently discovered PP4 and PP6 enzymes which belong to the PP2A subfamily, as well as a related PP5 enzyme (reviewed in ref 19). In fact, the PP5 phosphatase also consists of a conserved C-terminal catalytic domain and an N-terminal extension that participates in protein-protein interactions (53, 54). The localization of the phosphatase in the thylakoid membrane and the proteolytic release of a 34 kDa catalytic domain imply that the catalytic domain is exposed to the stroma and it is anchored to the membrane via a hydrophobic, possibly transmembrane stretch. The thylakoid phosphatase is present in very low abundance; it could only be detected serologically when highly purified and concentrated. The size of the catalytic subunit was identified to be either 41, 39, or 37 kDa, depending on the preparation. Perhaps there are two related PP2A-like phosphatases, or perhaps the phosphatase can assume various states due to partial proteolysis and/or other post-translational modifications such as phosphorylation. In support of this statement, the catalytic subunits of both PP1 and PP2A type phosphatases are known to undergo reversible phosphorylation (55, 56).

An important finding of our current work is that there is physical and functional association between the cyclophilin TLP40 and the PP2A-like protein phosphatase. The thylakoid phosphatase consistently copurified with TLP40; this in fact is how TLP40 was discovered (37). TLP40 could be removed from the phosphatase catalytic subunit irreversibly bound to microcystin-agarose. Additionally, protein phosphatase activity co-immunoprecipitated with TLP40 (37), and sequence analysis revealed that TLP40 contains phosphatase binding

epitopes. Before this discovery, only calcineurin (PP2B), a protein not considered to be present in plants (57), was known to bind cyclophilins (20–23). Moreover, PP2B enzymes interact with cyclophilins only in the presence of the exogenous drug CsA. The drug was not required for the phosphatase-TLP40 interaction we observed; however, thylakoid protein dephosphorylation was activated by CsA treatment, indicating a functional connection between the phosphatase and TLP40. Furthermore, phosphatase activation occurred only when CsA had access to TLP40 and at CsA concentrations which inhibit the PPIase activity of the purified TLP40. The protein phosphatase could be inhibited by adding prolyl-containing peptides, substrates of PPIase activity, to ruptured thylakoids in which the peptides had access to the luminal TLP40. On the basis of these observations, we propose that thylakoid protein dephosphorylation is regulated by the luminal TLP40 PPIase via a signaling pathway across the thylakoid membrane, directed from the inside to the outside of the lipid bilayer. The phosphatase may be regulated by the reversible binding of TLP40 to a transmembrane protein which could be the phosphatase itself or an accessory transmembrane protein(s) which anchors the phosphatase to the TLP40 on the opposite side of the thylakoid membrane.

We found that TLP40 associates with the inner thylakoid membrane surface. It is conceivable that membrane phosphatase activity is suppressed when TLP40 binds to a site on the inner thylakoid surface and stimulated when TLP40 is released into the lumen. Our recent observations (58) that heat shock caused significant activation of the membrane protein phosphatase and release of TLP40 into the thylakoid lumen are consistent with this hypothesis. The activation of protein dephosphorylation by CsA is likely caused by formation of the CsA-TLP40 complex which prevents TLP40 from associating with the membrane. A similar mechanism appears to operate to open or close the mitochondrial permeability transition pore (MTP) (59). Binding of the matrix cyclophilin CyP-M to the MTP leads to pore opening, while dissociation results in pore closure (59, 60). Furthermore, upon addition of CsA, CyP-M is released from the membrane pore into the matrix (59–61).

The N-terminal domain of TLP40 contains several putative protein-protein interaction modules (37) and likely contains a membrane-anchoring domain. Two potential phosphatase binding modules that flank two leucine zipper consensus sequences share high degrees of sequence homology with amino acid sequences in the proteins which bind PP2A (Figure 8). Analogous short motifs in the regulatory proteins were characterized to be important for binding to the phosphatases of the PPP family (45, 46, 48, 49, 62). We propose that TLP40 can only bind to the membrane phosphatase after the two binding modules have been brought together by an intramolecular, antiparallel sealing of the two leucine zipper elements. The homologous binding loops in the PP2A regulatory subunit A are required to be close for them to bind the catalytic subunit (45). The spatial context of the two phosphatase binding modules could be controlled by the isomeric state of two proline residues in the first leucine zipper element of TLP40 (Figure 8). The cis-trans isomerization of these residues might be an autocatalytic process and may be influenced by prolyl-containing polypeptides competing for the TLP40 PPIase active site.

The thylakoid membrane phosphatase we purified was very efficient in dephosphorylating PSII phosphoproteins. In contrast, the previously isolated soluble phosphatases that associate with thylakoids were most active in dephosphorylating LHCII polypeptides (13, 14, 51). Thus, we propose that the primary physiological function of the thylakoid membrane phosphatase we isolated in this study is to dephosphorylate PSII phosphoproteins. Under photoinhibitory stress conditions, PSII reaction center subunits are turned over rapidly, especially the D1 protein (1, 63, 64). This process is controlled by protein dephosphorylation; photo-damaged D1 and D2 proteins must be dephosphorylated to be degraded (5). Only after removal of the damaged protein can a new copy of D1 be inserted into the PSII complex, thereby restoring photosynthetic activity (1, 5). The TLP40 PPIase is believed to catalyze the folding of prolyl-containing proteins upon insertion into the thylakoid membrane or translocation into the thylakoid lumen (37). Thus, protein degradation, protein folding, and phosphatase activity are interdependent. High concentrations of unfolded polypeptides in the thylakoid lumen would suppress the phosphatase activity, just like we found in this study. This, in turn, would retard degradation of membrane phosphoproteins. Completion of protein folding would accelerate the dephosphorylation of damaged proteins, trigger their degradation, and allow for the introduction of the newly synthesized thylakoid polypeptides. We therefore propose that transmembrane signaling from TLP40 to the protein phosphatase coordinates protein folding inside the thylakoid lumen with protein dephosphorylation at the outer surface and thereby controls the protein turnover process.

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