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pCyP B: A Chloroplast-Localized, Heat Shock-Responsive Cyclophilin from Fava Bean

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When the immunosuppressants cyclosporin A (CsA) and FK506 bind to their intracellular receptors (immunophilins), they form complexes that bind to calcineurin and block calcineurin-dependent signaling pathways in immune cells. Previously, we reported that higher plants also express immunophilins and have a Ca^{2+}-dependent signaling pathway sensitive to immunophilin-ligand complexes. Based on an N-terminal peptide sequence of a chloroplast-localized cyclophilin (pCyP B), we isolated a cDNA clone encoding the preprotein of the cyclophilin. The deduced amino acid sequence of this cDNA starts with a putative transit sequence for chloroplast targeting. The mature pCyP B protein has rotamase activity with low-substrate specificity. Enzyme activity was inhibited by CsA with an inhibition constant of 3.9 nM. Similar to other CyPs from mammalian cells, pCyP B, when complexed with CsA, inhibited the phosphatase activity of bovine calcineurin. The mRNA level of pCyP B was high in leaf tissues but was not detectable in roots. Expression of the transcript in the leaf tissues was regulated by light and induced by heat shock. These findings illustrate the conserved nature of cyclophilin proteins among all of the eukaryotes and suggest that cyclophilins have a unique mode of regulation in higher plants.

INTRODUCTION

Immunophilins consist of two families of intracellular receptors for the immunosuppressants cyclosporin A (CsA), FK506, and rapamycin (Schreiber, 1991). CsA binds to cyclophilins, and FK506 and rapamycin bind to FKBPs (FK 506 binding proteins). The cyclophilin A–CsA and FKBP12–FK506 complexes bind to and inhibit the activity of calcineurin, thereby blocking a Ca^{2+}-dependent signal transduction pathway in a variety of cells, including human T lymphocytes (Liu et al., 1991; Clipstone and Crabtree, 1992; O'Keefe et al., 1992), yeast (Foor et al., 1992), and plant guard cells (Luan et al., 1993).

In addition to their role as coinhibitors of calcineurin-dependent signaling pathways in the presence of the natural products, immunophilins also catalyze the interconversion of *cis*- and *trans*-rotamers of peptidyl-prolyl amide bonds (Fischer et al., 1989; Harding et al., 1989). These rotamases accelerate the refolding of certain proline-containing proteins in vitro and apparently in vivo (Gething and Sambrook, 1992), suggesting a role for immunophilins in intracellular protein folding. CsA is a potent inhibitor of the rotamase activity of cylophilins and of collagen triple helix assembly in fibroblasts (Steimann et al., 1991). In addition, CsA inhibits the formation of the correct form of transferrin in liver cells (Lodish and Kong, 1991). It is not possible, however, to know whether this correlation is due to an important role for the enzymatic activity or whether the enzyme active site is playing the role of a receptor that can bind to unfolded intermediates of protein substrates. A possible chaperone role for immunophilins is suggested by studies of a cyclophilin homolog NinaA in fruit flies that is required for the transit of specific isoforms of rhodopsin from the endoplasmic reticulum (Stamnes et al., 1991). Protein folding studies with carbonic anhydrase also indicate that cyclophilins can function as chaperones (Freskgard et al., 1992). This possibility is supported by a recent report (Sykes et al., 1993) that demonstrated the heat shock–responsive expression of cyclophilin mRNAs in yeast.

To date, immunophilin homologs have been found in organisms ranging from bacteria to mammals (for review, see Schreiber, 1991; Rosen and Schreiber, 1992). In higher plants, Gasser et al. (1990) cloned a cyclophilin homolog (Rot1) that is ubiquitously expressed in a variety of plant tissues. By using affinity chromatography, we found several immunophilins from fava bean plants (Luan et al., 1993). Our studies also demonstrated that a CsA-sensitive, Ca^{2+}-dependent signaling pathway is present in the guard cells of this plant (Luan et al., 1993). To further characterize the immunophilins and to dissect the components of immunophilin-sensitive signaling pathways, we have purified and determined the subcellular localization of several immunophilins, including two cyclophilins. One (pCyP A) is the cytosolic form expressed in both root and leaf tissues (a Rot1 homolog), and the other (pCyP B) is specifically expressed in green tissues and is localized in the chloroplast (Luan et al., 1994).

We now report the isolation and characterization of a cDNA that encodes the precursor form of pCyP B. The amino acid

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sequence deduced from the cDNA contains a presequence of amphiphilic nature typical of a transit peptide for chloroplast translocation. We also show that the recombinant pCyP B protein has a CsA-sensitive rotamase activity. In the presence of CsA, pCyP B inhibited the phosphatase activity of bovine calcineurin. This supports our previous suggestion that CsA inhibits a calcineurin-like protein phosphatase in plant cells by first binding to an endogenous CyP (Luan et al., 1993). The expression of pCyP B was regulated by tissue specificity and by a light signal. The mRNA level of pCyP B was also responsive to heat shock, suggesting a possible function for this cyclophilin under stress conditions.

RESULTS

A Chloroplast-Localized Cyclophilin, pCyP B

As described in a previous report (Luan et al., 1994), we have detected two cyclophilin proteins from fava bean leaves that we named pCyP A and pCyPB/C. pCyPB/C is present in the chloroplast and mitochondrion. In this report, we refer to the two plant CyPs as pCyP A and pCyP B (i.e., pCyP B is synonymous with pCyPB/C). As shown in Figure 1, pCyP B is the only cyclophilin in the chloroplast that can be retained on a CsA affinity matrix. The N-terminal sequence of pCyP B protein was determined to be ASGAQGEVAELQAKVTSKIFFDIEIGGESAGRIV. The underlined region of this sequence was used to generate the oligonucleotide probe for the cDNA cloning of pCyP B.

Figure 1. Chloroplast Localization of the pCyP B Protein.

Cyclophilin proteins were purified by a CsA affinity matrix from chloroplast (lane 1) and from whole cell lysates (lane 2). After separation by SDS-PAGE, proteins were detected by silver staining. At left, the number indicates the molecular mass of pCyP B, which is 21 kD.

Isolation of a cDNA Encoding pCyP B Precursor Protein

A 41-mer degenerate oligonucleotide that was based on the peptide sequence described above was synthesized. The sequence of the probe is 5′-GGIGCACAAGGIGAAGTTGCAGAAYTICAAGCAAARGTTAC-3′. After screening the cDNA library with the probe, 28 positive clones were isolated. Six of the clones were purified and subcloned into the pEX*lox* phagemid by automatic transfer of the insert in the presence of *Escherichia coli* BM25.8 (Novagen, Madison, WI) according to the manufacturer's instructions. The inserts from the six clones were mapped by restriction digestion and shown to be identical. The nucleotide sequence of the longest insert was determined using the Sequenase II kit (U.S. Biochemicals) and is shown in Figure 2A. The cDNA is 1160 bp in length and contains an open reading frame of 744 bp that encodes a protein with 248 amino acids. By comparing the N-terminal sequence of the purified protein with the polypeptide sequence deduced from the cDNA, the cloned protein was shown to contain a 65–amino acid presequence that is presumably used to target the protein to the chloroplast. Because in-frame termination codons were present immediately upstream of the putative initiation codon, it is unlikely that this cDNA encodes larger preproteins.

Alignment of the mature pCyP B amino acid sequence with sequences of other CyP homologs reveals that pCyP B shares the highest degree of similarity with Rot1, a cytosolic CyP from higher plants (Figure 2B) (Gasser et al., 1990). In a sequence alignment with other CyPs, Gasser et al. (1990) noticed that the Rot1 sequences from three different plants all contain a seven–amino acid insertion relative to the mammalian or fungal CyPs. However, pCyP B does not have this insertion (Figure 2B, dashed lines).

pCyP B Is a CsA-Sensitive Rotamase

After cleavage from its fusion protein and purification (see Methods), the recombinant pCyP B protein sample was shown to yield a single band (21 kD) on a silver-stained SDS–polyacrylamide gel (Figure 3A). As shown in Figure 3B and Table 1, purified pCyP B had rotamase activity and showed little substrate specificity. CsA inhibited this activity with an inhibition constant (K_i) of 3.9 nM (Figure 3C).

Complex of pCyP B-CsA Inhibits Calcineurin Activity

Several cyclophilin–CsA complexes have been shown to inhibit the Ca^{2+} calmodulin-dependent protein phosphatase calcineurin and to block T cell activation (Swanson et al., 1992; Bram et al., 1993). In an earlier article (Luan et al., 1993), we reported that CsA blocks a Ca^{2+}-dependent signal transduction pathway in guard cells of fava bean. A Ca^{2+}-dependent, CsA-inhibitory protein phosphatase activity was also detected

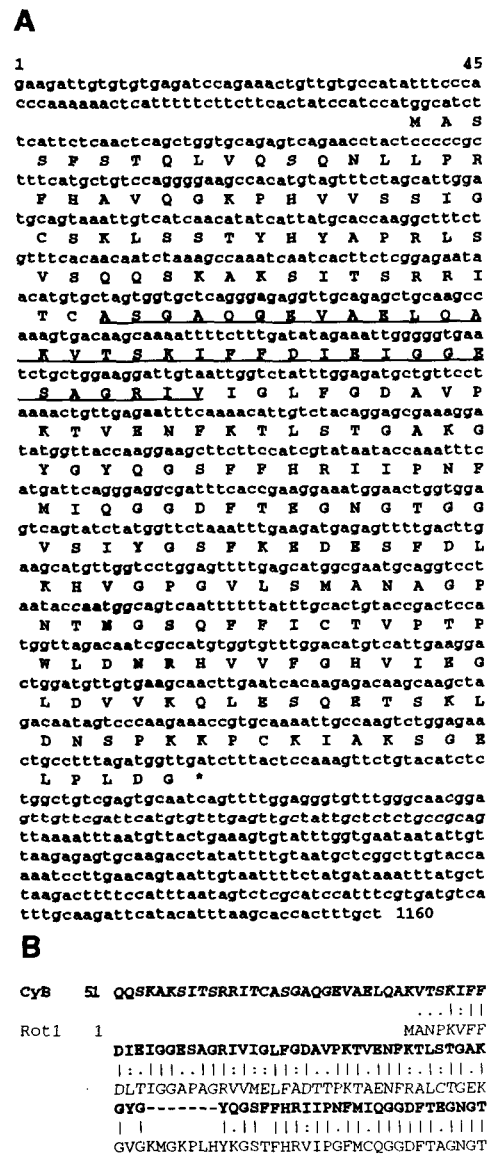

Figure 2. Sequence Analyses of a cDNA for pCyP B.

(A) Nucleotide sequence of a cDNA for pCyP B. The underlined region is the N-terminal sequence determined by Edman degradation of the purified protein. An asterisk represents the stop codon. The GenBank, EMBL, DDBJ accession number is L32095.

(B) Sequence alignment of pCyP B (CyB) with Rot1, the cytosolic form of cyclophilins from tomato. The dashed line indicates the deletion of the pCyP B sequence relative to Rot1. Vertical rules indicate identical amino acids; colons, conserved replacements; dots, different residues; asterisks, stop codons.

in protein extracts from this plant. We speculated that CsA binds to plant CyP(s) and thereby inhibits a calcineurin-like protein phosphatase. To confirm that plant CyPs can actually mediate the inhibitory effect of CsA on calcineurin, we assayed calcineurin activity in the presence of various concentrations of the pCyP B–CsA complex. As shown in Figure 4, pCyP B–CsA inhibited calcineurin activity with K_i of ~190 nM. This value is comparable to the potency of the complexes formed by CsA and mammalian CyPs (Swanson et al., 1992; Bram et al., 1993). Neither CsA nor pCyP B alone inhibited calcineurin activity (data not shown).

Expression of pCyP B mRNA Is Leaf Specific and Light Regulated

During the purification of pCyP B protein from fava bean plants, we noticed that the protein is more abundant in leaf tissues than in roots (Luan et al., 1994). To determine whether the expression is regulated at the mRNA level, we analyzed the pCyP B transcript in leaf and root tissues of fava bean plants. As shown in Figure 5A, the mRNA level was high in leaf tissues but not detectable in roots. We also found that green leaves produce more pCyP B protein than etiolated leaves (Luan et al., 1994). When mRNA levels in etiolated and greening leaves were studied, we found that pCyP B transcripts accumulated in a light-dependent manner (Figure 5B).

Transcript Level of pCyP B Is Elevated by Heat Shock Treatments

It has been shown that in yeast the transcription of CyP genes is induced by heat shock (Sykes et al., 1993). To test whether the chloroplast-localized CyP in fava bean is responsive to heat shock, we transferred the plants to a growth chamber with the same light condition but at a higher temperature (37°C). As shown in previous studies (Ko et al., 1992; Wang et al., 1993), plants at this temperature increase the levels of mRNAs and proteins responsive to heat shock. After heat treatment, mRNA levels of pCyP B in the control and treated leaves were analyzed by RNA gel blot. As shown in Figure 6, heat shock significantly increased the accumulation of pCyP B transcript (three- to fivefold).

DISCUSSION

Recently, we reported the isolation of several immunophilins from a higher plant, the fava bean. To characterize the cellular function of these plant proteins, we determined the cellular and subcellular distribution of both FKBPs and cyclophilins. The chloroplast has at least two immunophilins, a 13-kD FKBP and a 21-kD cyclophilin, pCyP B (previously referred to as pCyPB/C) (Luan et al., 1994). We now report the cloning and

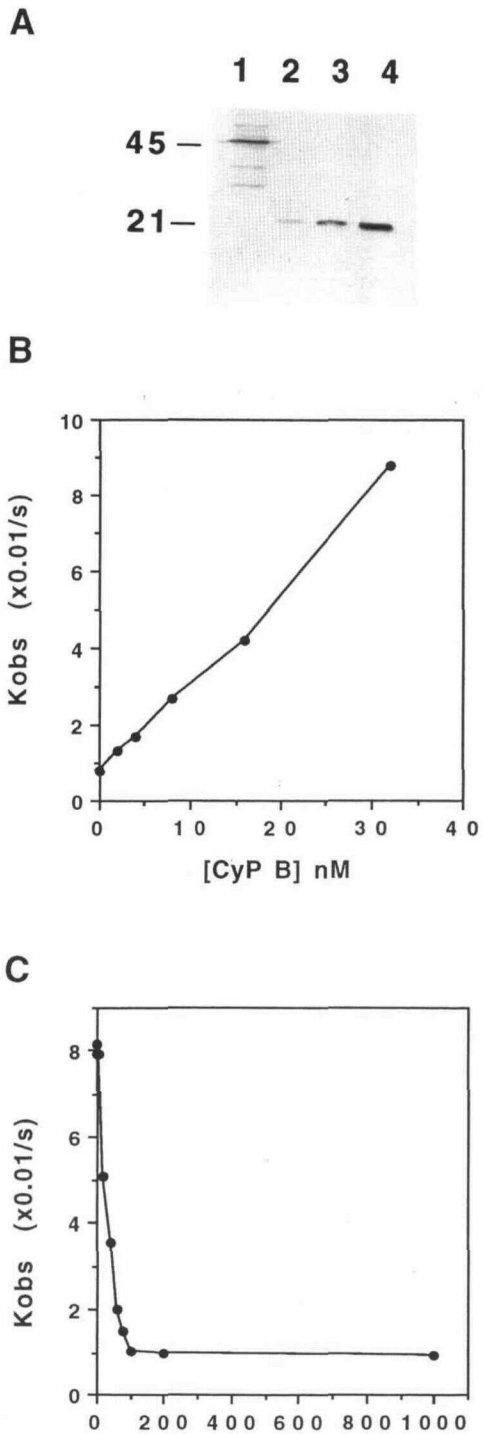

Figure 3. Characterization of the Recombinant pCyP B Protein.

(A) Purification of recombinant pCyP B from *E. coli*. The GST–pCyP B fusion protein (lane 1) and purified recombinant pCyP B (lanes 2 to 4 with 0.1, 0.3, and 1.0 μg of protein, respectively) were analyzed by SDS-PAGE and detected by silver staining. The molecular masses of pCyP B (21 kD) and GST–pCyP B (45 kD) are indicated at left.

Table 1. Substrate Specificity of pCyP B

Xaa[1]	K_{obs}/K_{unc}[2]
Ala	12
Val	8
Leu	7
Nle	9
Ile	4
Glu	2
Phe	2

[1] The assay substrate is succinyl-Ala-Xaa-Pro-Phe-p-nitroanilide. [2] K_{obs} is the rate constant in the presence of 20 nM pCyP B. K_{unc} is the rate constant without pCyP B.

characterization of the cDNA for pCyP B. The core region of the deduced amino acid sequence is highly conserved (50 to 70% identity) in a comparison to CyPs from mammalian and fungal systems. Although the mature pCyP B sequence is most homologous to the plant CyP homolog Rot1 (Gasser et al., 1990), pCyP B contains a 65–amino acid presequence with an amphiphilic composition that is most likely the transit peptide for chloroplast targeting. It is also noteworthy that Rot1 has an intriguing seven–amino acid insertion relative to pCyP B and other CyPs. This inserted region is positively charged due to the presence of two lysine residues (Gasser et al., 1990). It will be interesting to determine whether this insertion is functionally important.

The conservation of the cyclophilin proteins is also reflected by the rotamase activity they have retained during evolution. By using purified recombinant protein, we have demonstrated conclusively that pCyP B is a rotamase enzyme. In comparison to mammalian CyPs, the specific catalytic activity of pCyP B is approximately five times lower, but the inhibition constant for CsA (3.9 nM) is virtually the same. As protein foldases, cyclophilins are implicated in the protein folding pathway in living cells. Recent studies using an in vitro system have shown that cyclophilins serve as a chaperone in addition to their role as a foldase during the folding processes (Freskgard et al., 1992). In this report, we have characterized a cyclophilin that is localized in the chloroplast. Most of the chloroplast proteins are synthesized in the cytoplasm and translocated into the organelle after translation (Keegstra, 1989). It is believed that proteins are transported in unfolded forms across the membrane channels (Glick and Schatz, 1991). The imported proteins in chloroplasts require refolding (Gatenby and

(B) Rotamase activity of pCyP B. Enzyme assays were performed using succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as substrate in the presence of different concentrations of the pCyP B protein.

(C) CsA inhibits rotamase activity of pCyP B. Rotamase activity was determined using the same substrate as given in **(B)** with varying concentrations of CsA(nM). The concentration of pCyP B was 20 nM. Kobs is as given in Table 1; s, seconds.

Ellis, 1990) before they can function, for example, in the photosynthetic processes. The rotamase activity of pCyP B is consistent with a potential role for this protein in the refolding process of chloroplast proteins.

This notion is further supported by the heat shock–responsive expression of pCyP B mRNA. Recently, heat shock protein 70 (Hsp70) has been isolated and characterized from the chloroplast (Amir-Shapira et al., 1990; Marshall et al., 1990). Although its function has not been identified in this organelle, Hsp70 has been shown to facilitate the import and folding of proteins that enter the mitochondria (Glick and Schatz, 1991). When comparing Hsp70 and cyclophilin families, striking similarities are found in their expression pattern, distribution, and conservation during evolution. Both are highly expressed and are present in all cellular compartments. Each family has members that are induced by heat shock, suggesting a role under stress conditions. Proteins from both families are found in organisms ranging from bacteria to mammals and have amino acid sequences that are highly conserved. In fact, both a 40-kD cyclophilin and a 59-kD FKBP have been found to associate with Hsp70 and Hsp90 in the unactivated estrogen receptor complex (Tai et al., 1992; Ratajczak et al., 1993) through contact with the regions that share sequence identity in CyP40 and FKBP59. Studies of the functional significance of these associations will provide further information on the relationship among Hsp70, Hsp90, and immunophilins.

Following the discovery of calcineurin as a common target for CsA and FK506, we used these natural products as diagnostic probes to study calcineurin-mediated processes in plant cells (Luan et al., 1993). We demonstrated that CsA blocks the

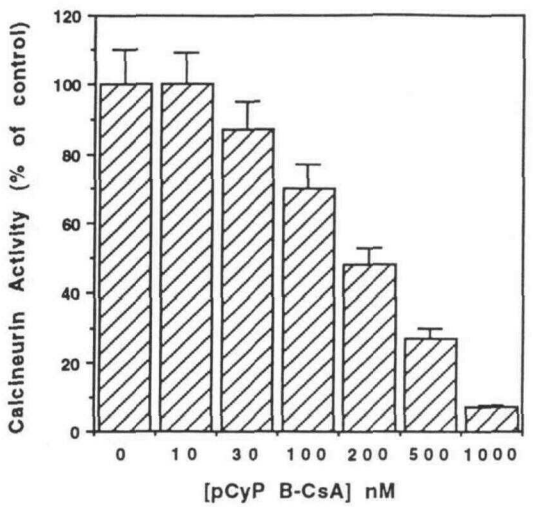

Figure 4. Inhibition of Calcineurin Activity by the pCyP B–CsA Complex.

Calcineurin assays were performed in the presence of different concentrations of the preformed pCyP B–CsA complex. The relative activity is presented as a percentage of control activity (in the absence of pCyP B–CsA). The concentration of bovine calcineurin was 100 nM.

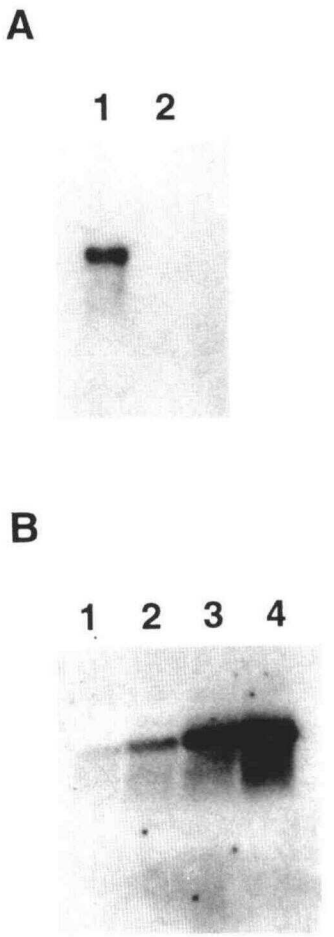

Figure 5. Tissue-Specific and Light-Regulated Accumulation of pCyP B mRNA.

(A) Tissue-specific expression of pCyP B mRNA. Total RNA (20 μg) from leaves (lane 1) and roots (lane 2) was separated on a 1.2% agarose gel, and the rRNAs were stained with ethidium bromide to indicate that equal amounts of total RNA were loaded into each lane. The mRNA levels of pCyP B were analyzed by RNA hybridization using pCyP B cDNA as the probe (see Methods).

(B) Light-inducible expression of pCyP B mRNA. Total RNA (20 μg) samples from leaves that were etiolated (lane 1) and illuminated (for 6 [lane 2], 16 [lane 3], and 40 hr [lane 4]) were separated on an agarose–formaldehyde gel and analyzed by RNA gel blotting.

Ca^{2+}-dependent K^+ channel inactivation in guard cells by inhibiting a calcineurin-like protein phosphatase. Because only the CyP–CsA complexes but not the separate components alone inhibit calcineurin activity, we hypothesized that CsA forms receptor–ligand complexes with the endogenous CyPs in plant cells before it can interfere with the Ca^{2+} signaling. Here, we present data showing that a plant CyP protein retains the ability to inhibit calcineurin activity in the presence of CsA. Although the cytosolic form of plant CyPs should be

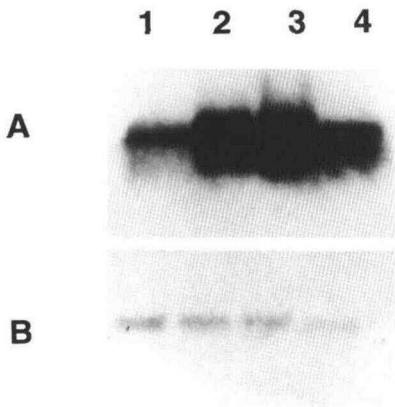

Figure 6. Heat Shock–Responsive Accumulation of the pCyP B Transcript.

(A) Total RNA (20 μg) from plant leaves grown at 23°C (lane 1) or grown at 23°C and then transferred to 37°C for 45 min (lane 2), 2 hr (lane 3), and 4 hr (lane 4) was analyzed by RNA gel blotting.
(B) The mRNA levels of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (which is not heat responsive) are shown as a standard.
The relative density of the pCyP B bands were quantified by a laser beam scanner as 1.0 (lane 1), 4.7 (lane 2), 4.5 (lane 3), and 1.9 (lane 4).

responsible for the effects that we observed previously (Luan et al., 1993), the high homology between pCyP B and Rot1 almost certainly confers the same inhibitory ability to Rot1 (or pCyP A) against calcineurin. To date, all of the CyP proteins studied form complexes with CsA that inhibit the phosphatase activity of calcineurin. The conservation of calcineurin inhibition by CyP in mammalian, yeast, and plant cells suggests the possibility that CyPs play a role in the signal transduction pathways in these eukaryotic cells, although an endogenous compound with CsA-like properties has yet to be identified.

METHODS

Purification of the Chloroplast-Localized Cyclophilin

Chloroplasts were isolated from 14-day-old seedlings of fava bean plants according to the procedure described by Price et al. (1987). They were lysed by sonication in the extraction buffer (50 mM Tris, pH 7.5, 4 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/mL leupeptin). The homogenate was centrifuged at 100,000*g* for 45 min in a Beckman SW40 rotor. The supernatant was adjusted to contain 150 mM NaCl and then loaded onto an affinity column packed with cyclosporin A (CsA)–Affigel matrix (Bio-Rad) (Fretz et al., 1991). Purified cyclophilins were separated by SDS-PAGE and transferred to Problot membrane (Applied Biosystems Inc., Foster City, CA). The N-terminal sequence of the protein was determined by automated Edman degradation.

Construction and Screening of a Fava Bean cDNA Library

Total RNA was isolated from leaf tissues of fava bean plants. Leaves of 14-day-old fava bean plants were frozen in liquid nitrogen and ground into a fine powder in a mortar. The tissue powder was transferred to a test tube containing an equal volume mixture of phenol and lysis buffer (100 mM Tris, pH 7.5, 100 mM LiCl, 10 mM EDTA, and 1% SDS) and mixed by vortexing for 20 sec. Chloroform was then added to the homogenate, and the mixture was vortexed for another 30 sec. After 10 min of centrifugation at 6000*g*, the upper phase was transferred to a clean tube and total nucleic acid (DNA/RNA) was precipated with isopropanol. Total RNA was separated from DNA by precipitation in 2 M LiCl. Poly(A)$^+$ RNA was purified by the PolyATract mRNA isolation system from Promega. The cDNAs were synthesized using a cDNA synthesis kit from Boehringer Mannheim according to the instructions from the manufacturer. The cDNA library was constructed in the LamdaEX*lox* vector (Novagen, Madison, WI). A library with 200,000 phage was obtained; the procedure described in Sambrook et al. (1989) was used to screen the library. According to the codon usage of fava bean genes, the peptide sequence GAQGEVAELQAKVT was used to design a low-degeneracy oligonucleotide probe: 5′-GGIGCACAAGGIGAAGTTGCAGAAYTICAAGCAAARGTTAC-3′. The probe was synthesized on a DNA synthesizer (Millipore, Bedford, MA) and was ^{32}P-labeled by T4 polynucleotide kinase. The labeled probe was purified by the Push-through Column (Stratagene) and added to the hybridization buffer. The hybridization was performed at 42°C for 12 to 18 hr. The filters were washed three times in 0.2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.5% SDS for 30 min each, and the air-dried filters were exposed to Kodak XAR-50 x-ray film at −78°C with an intensifying screen.

RNA Blotting and Hybidization

RNA samples (total RNA isolated as described above) were separated by electrophoresis through a 1.2% agarose–formadehyde gel and blotted onto the Zeta probe membrane (Bio-Rad) in 0.05 M NaOH. The membrane was neutralized in 0.5 M Na_2HPO_4, pH 7.2, and prehybridized in 7% SDS, 0.5 M Na_2HPO_4, pH 7.2, and 1 mM EDTA for 30 min at 60°C. The randomly labeled cDNA was denatured by 0.2 M NaOH for 15 min at room temperature and added to the prehybridization buffer. The hybridization was maintained at 60°C for 12 hr before the membrane was washed in 40 mM Na_2HPO_4, pH 7.2, and 0.5% SDS. Filters were air dried and exposed to the x-ray film as described above.

Expression of Recombinant pCyP B Protein in *Escherichia coli*

The cDNA fragment encoding the mature protein of the cyclophilin pCyPB/C was amplified by polymerase chain reaction and cloned in frame with glutathione *S*-transferase (GST) into the pGEX3x vector (Pharmacia). *E. coli* (BL21) cells transformed with the plasmid harboring the fusion protein construct were grown to log phase, and expression of the fusion protein was induced by addition of 0.4 mM isopropyl-β-D-thiogalactoside to the medium. Cells were harvested 12 hr after induction and resuspended in PBS buffer. Purification of the GST fusion protein was performed essentially as described by Smith and Johnson (1988). After lysis by sonication, the suspension was centrifuged at 10,000*g* for 15 min. The supernatant was incubated with glutathione Sepharose 4B (Pharmacia) (2-mL bed volume per 1 L of

culture) for 30 min at 4°C on a rotating platform. The glutathione beads were washed three times with PBS, and the fusion protein was eluted by 5 mM reduced glutathione in 50 mM Tris buffer, pH 8.0. The eluate was dialyzed in 4 L of 50 mM Tris buffer, pH 8.0, for 24 hr. The cleavage reaction was performed in a buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, and 2 mM $CaCl_2$. Factor Xa (Boehringer Mannheim) was used as the endoprotease at a concentration of 0.5 to 1% (relative to the amount of fusion protein in the reaction). After 5 hr at 25°C, the reaction mixture was loaded on a glutathione Sepharose column. GST and the uncleaved fusion protein were retained on the column, and the pure recombinant pCyP B protein was retained in the effluent.

Rotamase Assays

The rotamase activity of recombinant pCyP B was determined using several synthetic peptides as substrates (Table 1) in a two-step, coupled reaction involving chymotrypsin, as described previously by Fischer et al. (1989). All assays were performed in 1.5-mL samples that contained 50 mM Hepes, pH 7.9, 60 μM of substrate peptide, 40 nM pCyP B, and varying concentrations of CsA. The reactions were initiated by adding 3.5 mg of chymotrypsin (Sigma) and were monitored by measuring absorbence at 408 to 412 nm for 10 min with a UV/vis spectraphotometer. Data analyses were performed according to the programs written by Standaert (1992).

Calcineurin Inhibition by pCyP B-CsA Complex

Calcineurin assays were performed as described previously (Manalan and Klee, 1983; Liu et al., 1992) with a few modifications. Briefly, a peptide substrate (DLDVPIPGRFDRRVSVAAE) was ^{32}P-labeled at the serine residue by the catalytic subunit of protein kinase A (Sigma) and ^{32}P-ATP. The labeled peptide was separated from free ATP by a Sep-Pak18 column (Millipore). For calcineurin assays, 100 nM each of calcineurin and calmodulin (both from Sigma) were included in the assay buffer (50 mM Tris, pH 7.5, 6 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM DTT, 100 μg/mL BSA) in the presence of varying concentrations of the pCyP B–CsA complex. pCyP B and CsA were premixed at a molar ratio of 1:10 and incubated at 4°C for 2 hr before they were added to the calcineurin-containing buffer. The mixture (a total volume of 50 μL) was incubated for another 20 min at 4°C, and the reaction was started by adding 10 μL of labeled peptide substrate (final concentration of 1 μM). The reactions were performed at 30°C for 10 min and terminated by the addition of 500 μL of a stop solution (20% trichloroacetic acid and 0.1 M K_2HPO_4). The cleaved ^{32}P-phosphate was separated from the peptide by a column packed with AG 50W-8X resin (Bio-Rad) and counted on a Beckman LS1801 system.

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