Partial Purification and Characterization of a Ca\textsuperscript{2+}-Dependent Protein Kinase from Pea Nuclei\textsuperscript{1}

Haimin Li, Marianne Dauwalder, and Stanley J. Roux*

Department of Botany, The University of Texas at Austin, Austin, Texas 78713

ABSTRACT

Almost all the Ca\textsuperscript{2+}-dependent protein kinase activity in nuclei purified from etiolated pea (Pisum sativum, L.) plumules is present in a single enzyme that can be extracted from chromatin by 0.3 molar NaCl. This protein kinase can be further purified 80,000-fold by salt fractionation and high performance liquid chromatography, after which it has a high specific activity of about 100 picomoles per minute per micogram in the presence of Ca\textsuperscript{2+} and reaches half-maximal activation at about 3 x 10\textsuperscript{-7} molar free Ca\textsuperscript{2+} without calmodulin. It is a monomer with a molecular weight near 90,000. It can efficiently use histone III-S, ribosomal S6 protein, and casein as artificial substrates, but it phosphorylates pheosvitin only weakly. Its Ca\textsuperscript{2+}-dependent kinase activity is half-maximally inhibited by 0.1 millimolar chlorpromazine, by 35 nanomolar K-252a and by 7 nanomolar staurosporine. It is insensitive to sphingosine, an inhibitor of protein kinase C, and to basic polypeptides that block other Ca\textsuperscript{2+}-dependent protein kinases. It is not stimulated by exogenous phospholipids or fatty acids. In intact isolated pea nuclei it preferentially phosphorylates several chromatin-associated proteins, with the most phosphorylated protein band being near the same molecular weight (43,000) as a nuclear protein substrate whose phosphorylation has been reported to be stimulated by phytochrome in a calcium-dependent fashion.

Light-activated phytochrome can stimulate the phosphorylation of certain proteins in isolated pea nuclei (4). This effect is calcium-dependent and can be inhibited by chlorpromazine, an antagonist of calcium-binding proteins. Chlorpromazine and W\textsuperscript{7}, another inhibitor of calcium-binding proteins, can also block the promotive effects of phytochrome on mRNA accumulation for the Chl a/b protein (12). These observations led Lam et al. (12) to speculate that calcium-regulated protein kinases might help transduce the phytochrome signal into changes in gene expression. The idea that protein kinases are involved in the process of light-regulated gene expression is supported by recent reports which show that the activity of protein kinases regulate the binding of transacting factors to promoter regions of phytochrome-regulated genes (3).

The results summarized above provide a rationale for investigating protein kinases in plant nuclei that are regulated by Ca\textsuperscript{2+}. Trewavas’ group pioneered the study of nuclear protein phosphorylation in plants (27), and, as reviewed by Ranjeva and Boudet (21) and by Roux et al. (23), several different laboratories have purified and characterized Ca\textsuperscript{2+}-dependent protein kinases in plants. Recently, Suzuki and Verma reported there was calcium-regulated protein kinase activity in nuclei isolated from soybean nodules (25). However, the only previous report that characterized a specific Ca\textsuperscript{2+}-dependent protein kinase which was possibly of nuclear origin is that of Polya et al. (18). They found that a chromatin-containing fraction from wheat germ had a M\textsubscript{r} 90,000 protein kinase that was calcium dependent and could be stimulated twofold by high concentrations (4 \mu M) of calmodulin. This stimulation was later found to be due most likely to the binding of calmodulin to basic polypeptide inhibitors of the kinase (19). Because they had purified this kinase from a relatively crude fraction of wheat germ, Polya and colleagues could only speculate that it might be chromatin associated (17, 18).

In this report we describe the partial purification and characterization of a M\textsubscript{r} 90,000 protein kinase from the chromatin fraction of purified pea nuclei, and we compare and contrast its properties with those of the wheat germ protein kinase isolated by Polya and colleagues (17, 18). We also provide here some documentation on the nuclear origin of the pea Ca\textsuperscript{2+}-dependent kinase.

MATERIALS AND METHODS

Plant Growth

Seedlings of pea, Pisum sativum L., cv Alaska, were grown in the dark for 7 d at 23 ± 3°C.

Materials

Percoll, ATP (Tris salt), PMSF, fluphenazine, chlorpromazine, dephosphorylated casein, calf thymus histones (type III-S), poly-L-lysine (mol wt 150,000–300,000), poly-L-arginine (mol wt 150,000–300,000), W\textsubscript{5}, W\textsubscript{7} were all obtained from Sigma Co. Linoleic acid was from Supelco Co. (Bellefonte, PA). Phosphatase labeled anti-guinea pig IgG was from Kierkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Staurosporine and K252a were from Kamiya Biomedical Corporation (Thousand Oaks, CA). The [\gamma\textsuperscript{32}P]ATP (850 cpm/pmol) was obtained from New England Nuclear. Calmodulin was purified from oat seedlings as previously described (1). Myelin basic protein peptide 4–14 (30) was synthesized on an Applied Biosystems automated solid phase
peptide synthesizer by the Protein Sequencing Center of the University of Texas at Austin, and then purified by reverse phase HPLC on a Beckman C-18 column. Purified 40 S ribosomes from rabbit reticulocytes were generously provided by Dr. G. Kramer from the laboratory of Dr. B. Hardesty (University of Texas, Austin). All buffers were prepared with water purified by a Milli Q water purification system (Millipore Corp.).

Preparation of Nuclei and Chromatin Fraction

Isolation of nuclei from the plumes of 7-d-old etiolated pea seedlings was done as described by Datta et al. (4). Typically, 80 g of plumes was used as starting material. The chromatin fraction was prepared from purified nuclei as described by Chen et al. (2). Briefly, gradient purified nuclei were lysed by incubating them for 10 min in 5 mL of buffer I (60 mM Hepes [pH 7.5], 1 mM EDTA, 0.5% Triton X-100, 0.5 mM PMSF in dimethyl sulfoxide, 1.14 mM ascorbic acid, 0.2 mM ammonium molybdate, 10 mM dithiothreitol), and then the extract was centrifuged at 9000 g for 1 min. The pellet of this centrifugation was referred to as the chromatin fraction.

Extraction and Column Chromatography of the Protein Kinase

All procedures were carried out at 4°C except the column chromatography steps, which were done at room temperature (22 ± 3°C). The chromatin fraction was gently shaken for 1 min in 5 mL of buffer II (same as buffer I, but without EDTA and Triton X-100), transferred into 2 mL of buffer III (= buffer II + 0.3 M NaCl, and 10 mM 2-mercaptoethanol in place of dithiothreitol), stirred for 20 min, then centrifuged at 9000 g for 5 min. The supernatant was removed and made 30% saturated ammonium sulfate by adding solid crystals of the salt. The solution was stirred for 20 min after the ammonium sulfate was dissolved, then centrifuged at 9000 g for 15 min. The supernatant was removed and its ammonium sulfate concentration was raised to 80% saturation. After being stirred for 20 min, the solution was centrifuged at 9000 g for 15 min. The supernatant was discarded and the pellet was dissolved in 0.5 mL of buffer A (30 mM Hepes [pH 7.5], 3 mM MgCl₂). The protein solution was clarified by centrifugation (9000 g for 5 min), then applied to a DEAE-5PW column (7.5 mm x 7.5 cm, Beckman Instruments, San Ramon, CA) that had been previously equilibrated with buffer A. Unbound proteins were eluted with 1.5 column volumes of buffer A, then bound proteins were step-eluted with 30 mM Hepes (pH 7.5), 3 mM MgCl₂, 0.9 M NaCl. Fractions with protein kinase activity were combined and CaCl₂ was added to a final concentration of 2 mM. This pooled fraction was applied to a HPLC polypropyl aspartamide column (4.6 x 100 mm, Poly LC, Columbia, MD). This column was equilibrated in a buffer containing 25 mM Tris-HCl (pH 7.5), 0.2 mM CaCl₂, 10 mM 2-mercaptoethanol. The column was washed with this same buffer to remove unbound proteins, then proteins whose binding was calcium-dependent were eluted with a buffer containing 25 mM Tris-HCl (pH 7.5), 2 mM EGTA, and assayed for protein kinase activity. Preparations purified through the polypropyl aspartamide column will be referred to as purified protein kinase.

To assess the native molecular weight of the protein kinase, peak activity fractions from the DEAE column were concentrated by an Amicon Centricon-10 concentrator and chromatographed on a calibrated Superose 12 HR 10/30 FPLC gel filtration column (10 x 300 mm, Pharmacia LKB Biotechnology Inc., Piscataway, NJ) equilibrated in a 10 mM sodium phosphate buffer with 150 mM NaCl (pH 7.0).

Protein Kinase Assays

Quantitative assays of protein kinase activity were made by measuring the incorporation of 32P from [γ-32P]ATP into histone HI type III-S. This assay was carried out at room temperature, following procedures described by Roskoski (22). The assay reaction was started by adding 10 μL enzyme solution to 40 μL of a reaction buffer, giving a final concentration of 25 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 2 mM EGTA, 0.1 mM free Ca²⁺, 1 mg/mL histone, 100 μM [γ-32P] ATP (850 cpm/pmol). The free Ca²⁺ concentration was calculated by a computer program based on the method of Perrin and Sayce (16). Following incubation of the assay mixture for 15 min, 30 μL was withdrawn and spotted onto 1 x 2 cm phosphocellulose filter strips (Whatman P81). The filter strips were washed sequentially with 75 mM phosphoric acid three times (5 min each wash), 95% ethanol (1 min), and ethyl ether (1 min). Each filter was air dried then added to a scintillation vial containing 10 mL aqueous counting scintillator (Amersham) and counted in a Packard scintillation counter.

The ability of purified protein kinase to phosphorylate the M₆₀,3,000 S6 protein from 40 S ribosomes was tested only qualitatively. The kinase preparation (10 μL, about 4 ng) was reacted with 25 μg of 40 S ribosomes in a final volume of 50 μL for 15 min. The sample was then mixed with SDS-PAGE sample buffer, boiled for 2 min, applied to a 10% SDS polyacrylamide gel, and electrophoresed as described by Laemmli (11). After SDS-PAGE the gel was fixed, stained, destained, dried, and autoradiographed as described in the next section. As a positive control, the 40 S ribosomes were also reacted under the same conditions with cAMP-dependent protein kinase, which is known to selectively phosphorylate the S6 protein in those ribosomes (28). Ribosomal proteins phosphorylated by each protein kinase were electrophoresed in adjacent lanes of the SDS-PAGE so that their relative mobilities could be directly compared.

To measure the effects of free calcium on protein kinase activity, the concentration of free calcium in the assay medium was set by using a Ca²⁺/EGTA buffer made up of variable concentrations of Ca²⁺ and a final concentration of 0.2 mM EGTA. The final free calcium concentration in the buffer was calculated by a computer program based on the method of Perrin and Sayce (16).

SDS-PAGE

Samples with the highest specific activity from the Polypropyl Aspartamide column were lyophilized by a Savant Speed Vac Evaporator, dissolved in water, then precipitated by TCA.
The pellet was washed by ethanol and ether, mixed with SDS-PAGE sample buffer, boiled for 2 min, applied to a 10% SDS polyacrylamide gel, and electrophoresed (11). After electrophoresis, protein bands were visualized by a silver stain method (15). When labeled samples were assayed, the phosphorilation reaction was carried out as described for the protein kinase assay. The reactions were stopped by boiling the samples in SDS-PAGE sample buffer. The gels were fixed with 15% TCA, 50% methanol, stained with Coomassie brilliant blue, destained, dried, and autoradiographed using Kodak AR film.

**Detection of Protein Kinase Activity in Gels after SDS-PAGE and Isoelectric Focusing**

These procedures were carried out as described by Guo and Roux (8). Briefly, the samples to be assayed after SDS-PAGE were heated to 80 to 90°C for 2 min in electrophoresis sample buffer and electrophoresed as described above, except that 1 mg/mL histone was included in the separating gel before polymerization. After electrophoresis, SDS was removed from the gel by washing it with buffer, then phosphorylation was initiated by incubating the gel in a reaction buffer containing [γ-32P]ATP (850 cpm/pmol) for 5 h as described (8).

For isoelectric focusing, the protein sample was concentrated and desalted by centrifugation in an Amicon Centricon-10 concentrator, applied to an isoelectric focusing agarose gel (pH range 3–10, FMC Products), focused, blotted onto nitrocellulose, and reacted with [γ-32P]ATP, all as described (8). Excess [γ-32P]ATP was removed by washing the blots in 5% TCA and 1% sodium pyrophosphate. The blots were air dried and labeled proteins were detected by autoradiography.

**Assay of Phosphorylated Amino Acids in Histone III-S**

The procedure used for this assay was similar to that described by Putnam-Evans et al. (20). [32P]Histone III-S that had been phosphorylated by reaction with the pea Ca2+-dependent protein kinase was hydrolyzed in 6 N HCl for 1 h at 110°C, dried under vacuum, resuspended in 10 μL of Milli-Q purified water and cochromatographed with phospho-amino acid standards (1 mg/mL each of D,L-phosphoserine, D,L-phosphothreonine, and D,L-phosphotyrosine) by paper electrophoresis (Whatman paper No. 1) in pyridine/acetic acid/water (5:50:945, v/v/v) at 2 kV for 90 min. The positions of the standards were visualized by spraying the paper with a ninhydrin spray: 0.05% ninhydrin in ethanol-acetic acid (3:1, v/v). The positions of the labeled amino acids were detected by autoradiography.

**Immunological Assays for Calmodulin and for 43 kD Substrate Protein**

Kinase samples purified through the DEAE column and through the Polypropyl Aspartamide column steps were assayed for calmodulin content by Western blot analysis, using anti-spinach calmodulin IgG given by Dr. L. Van Eldik (Vanderbilt University), as described by Guo and Roux (8). Oat calmodulin (1) was used as a positive control.

Antibodies to 43 kD nuclear protein that is phosphorylated by the Ca2+-dependent protein kinase were raised by Pocono Farms (Canadensis, PA) in guinea pigs. The antigen preparation used to inoculate the animals was Coomassie brilliant blue stained bands of the 43 kD protein excised from 10% polyacrylamide gels after SDS-PAGE of proteins extracted from pea chromatin by 0.3 M NaCl. Polyclonal IgG was purified from the serum of immunized guinea pigs by protein A affinity chromatography (14). Purified antibody was then used to: (a) immunoprecipitate the 32P-labeled 43 kD protein from a protein preparation extracted from pea chromatin by 0.3 M NaCl, using procedures described by Silberman et al. (24); (b) detect the 43 kD protein after Western blot analysis of salt-extracted proteins from pea chromatin, using procedures described by Guo and Roux (8); and (c) detect the 43 kD protein in nuclei squeezed from formaldehyde-fixed pea plumele cells, using the procedure for immunofluorescence staining of unembedded cells described by Dauwalder et al. (7). Nuclei released from the fixed cells were distinguished by their distinctive size and morphology as visualized under both brightfield and Nomarski optics.

**Protein Determination**

Protein concentrations were determined with a Bio-Rad protein assay kit using BSA as a standard.

**RESULTS**

**Nuclei and Chromatin Purification**

By light and electron microscopic examination, the purification of nuclei was judged to be free of contamination by other intact organelles (5). After extraction of the nuclei with Triton X-100 and EDTA, the remaining chromatin fraction contained nuclear scaffolding proteins in addition to the chromatin, but it was essentially free of the nuclear envelope and other membranes (data not shown).

**Purification of Protein Kinase**

A summary of the purification is presented in Table I. The initial 30% ammonium sulfate precipitation step does not improve the specific activity of the enzyme, but it helps to separate it from other protein kinases and from an NTPase.
that could compete with it for ATP (2, 6). All the Ca$^{2+}$-dependent kinase activity that can be eluted from the DEAE column emerges in the flow-through fraction of the column (Fig. 1A). Essentially all of the protein kinase activity in this fraction is Ca$^{2+}$-dependent. The DEAE-purified kinase binds to the polypropyl aspartamide column in a Ca$^{2+}$-dependent fashion, and its selective elution from the column by EGTA results in a many-fold increase in its purity (Fig. 1B; Table I). If all the silver-stained protein migrating at 90,000 M$\text{r}$ after SDS-PAGE is the kinase (Fig. 2, lane A), then its purity after the polypropyl aspartamide column would be near 20%.

**Molecular Weight and Isoelectric Point**

When the protein kinase activity was detected in the gel after SDS-PAGE, its position corresponded to a $M_r$ 90,000 protein band (Fig. 2, lane B). After isoelectric focusing, its activity appeared in two bands, pl 6.7 (major) and 6.4 (minor), on the gel (Fig. 2, lane C). Undenatured kinase also had a mol wt near 90,000 as estimated by molecular sieve chromatography (Fig. 1c).

![Figure 1](https://www.plantphysiol.org)  
**Figure 1.** Elution profiles of the $A_{280\text{ nm}}$ and of the protein kinase activity from DEAE-5PW column (A), hydrophobic interactive chromatography column (B), and Superose 12 HR 10/30 molecular sieve column (C). The numbers above the arrows in (C) are the mol wt $\times 10^{3}$ of the proteins used as standards, and the arrows mark the position of the elution peak of these standards. For all three columns, the volume of sample loaded was 0.25 mL, the flow rate was 0.5 mL/min, and the volume of each fraction collected was 0.2 mL.

![Figure 2](https://www.plantphysiol.org)  
**Figure 2.** A, Peak fraction of protein kinase activity from polypropyl aspartamide column after SDS-PAGE and silver staining. Total sample load was less than 200 ng. B, Same sample as in A. After SDS-PAGE, sample was renatured in the gel and reacted with $[^{32}\text{P}]$ATP. After washing out unbound label, the gel was dried and analyzed by autoradiography. The position of the one labeled band is at $M_r$ 90,000. C, Same sample as A. Sample was subjected to isoelectric focusing, transfer to nitrocellulose and reaction with $[^{32}\text{P}]$ATP as described in Datta et al. (5). Major autoradiographic signal is from band at pl 6.7, with minor signal at pl 6.4.

**Calcium Dependence**

The phosphorylation of histone III-S by the 90 kD protein kinase was highly dependent on Ca$^{2+}$. There was no detectable kinase activity in the absence of Ca$^{2+}$ (Fig. 3), but phosphorylation was markedly activated by free Ca$^{2+}$ concentrations above approximately 10$^{-7}$ M, with half-maximal activation reached at near 0.3 μM (Fig. 3). The same half-maximal activation concentration of Ca$^{2+}$ was found in two separate repeats of this assay. Linoleic acid did not shift the Ca$^{2+}$-dependence curve, nor did it increase the activity of the kinase in the presence or absence of Ca$^{2+}$ (Table II).

**Calmodulin Independence**

After purification on DEAE and polypropyl aspartamide columns, the protein kinase preparation contained no detectable calmodulin as judged by an immunoblot assay that could detect less than 20 ng calmodulin (Fig. 4). Adding 2 μM oat calmodulin to this preparation did not stimulate protein kinase activity (Table II). Calmodulin independence is also indicated by the presence of kinase activity in the 90 kD band after SDS-PAGE (Fig. 2B).

**Substrate Specificity and Localization of Endogenous Substrate**

The 90 kD nuclear kinase could use histone III-S and casein as substrates equally well, but it phosphorylated phosvitin poorly, and did not phosphorylate the myelin basic protein.
peptide (residues 4–14) that is favored by PKC (30) (Table III). Autoradiographic analysis of SDS-PAGE separated proteins from 40S ribosomes after their reaction with purified pea protein kinase indicated that the major band phosphorylated by the pea kinase has exactly the same Mr as the major band phosphorylated by cAMP-dependent protein kinase, which is known to be the S6 ribosomal protein (28) (data not shown).

When the hydrolysate of [γ-32P]histone was examined, approximately 90% of the labeled amino acids comigrated with phosphoserine and about 10% comigrated with phosphothreonine. There was no label at the phosphotyrosine position.

In highly purified nuclei preparations, the phosphorylation of several protein bands was stimulated by micromolar Ca2+, with a band near Mr, 43,000 showing the most enhanced phosphorylation (Fig. 5). As assayed by a Beckman DU-8B spectrophotometer equipped with a slab gel scanning apparatus, the autoradiographic density of the 43 kD band was enhanced threefold when the nuclei were labeled in the presence of 10 μM Ca2+, as compared to when they were labeled in 0.1 mM EGTA. In the absence of Ca2+, the Mr, 43,000 substrate can also be phosphorylated in pea nuclei by a spermine-stimulated protein kinase (H Li, SJ Roux, unpublished data). Antibodies to the Mr, 43,000 substrate strongly bound to nuclei teased from fixed but unembedded pea plumes under conditions in which IgG purified from preimmune serum showed little or no binding (Fig. 6).

### Inhibitor Profile

Using histone III-S as an artificial substrate, the concentration of half-maximal inhibition (Ki) of the purified nuclear kinase by a variety of protein kinase antagonists was determined. At least four different inhibitor concentrations were tested for each Ki determination. Staurosporine and K252a, which are strong antagonists of PKC (26, 29), were the most potent inhibitors of the pea nuclear kinase. On the other hand, spermine, which also strongly inhibits PKC (9), and basic polypeptides, which inhibit the wheat germ Ca2+-dependent kinase (19), were relatively ineffective against the pea nuclear kinase (Table II).

### DISCUSSION

The 90 kD protein kinase isolated from pea nuclei resembles the one isolated from wheat germ by Polyá and Davies.
Ca\textsuperscript{2+}-DEPENDENT KINASE FROM PEA NUCLEI

Table II. Comparison of Protein Substrates of the Ca\textsuperscript{2+}-Dependent Protein Kinase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone III-S</td>
<td>100</td>
</tr>
<tr>
<td>Dephosphorylated casein</td>
<td>96</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>20</td>
</tr>
<tr>
<td>Myelin basic protein peptide\textsubscript{a,14}</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Protein kinase activity was assayed in triplicate in the standard assay, as described in "Materials and Methods," using purified protein kinase and a concentration of 1 mg/mL of each substrate.

(17) in its native mol wt, its preferential phosphorylation of histone III-S and casein rather than phosvitin as artificial substrates, and its stimulation by micromolar calcium in the absence of calmodulin when histone III-S is used as the substrate. Also, like the wheat germ kinase, the pea kinase is extracted from a chromatin-containing fraction by NaCl concentrations \(\geq 0.3\, \text{M}\) and can be inhibited by phenothiazine compounds.

However, there are also significant differences. The wheat germ kinase is inhibited by basic polypeptides, and micromolar calmodulin can reverse this inhibition (19). The pea nuclear kinase activity is not affected by basic polypeptides or by micromolar added calmodulin, nor does it contain endogenous calmodulin when its histone phosphorylating activity is activated by Ca\textsuperscript{2+}. It also retains protein kinase activity after SDS-PAGE which would be expected to separate it from calmodulin. Linoleic acid lowers the Ca\textsuperscript{2+} concentration at which the wheat germ kinase shows half-maximal stimulation, and it stimulates the wheat germ kinase in the presence of calcium (13), but has no such effects on the pea nuclear kinase. The chromatin-containing fraction from which the wheat germ kinase was isolated was relatively impure: it was simply the pellet from a 40,000g centrifugation of a crude homogenate of wheat germ. The chromatin-containing fraction from which the pea kinase was extracted was derived from a highly purified fraction of pea nuclei that had been treated with Triton X and EDTA to remove the nuclear envelope and other associated membranes.

The evidence noted above indicates that the 90 kD pea kinase and 90 kD wheat germ kinase differ significantly from each other. However, some of the contrasting properties may be attributable to the different kinds of contaminating proteins present in the two kinase preparations when they were characterized, or to differences arising from the extraction conditions used. It cannot be excluded, then, that the 90 kD wheat germ kinase may be closely related in both structure and function to the pea nuclear kinase described here.

Several lines of evidence indicate that the pea protein kinase is a nuclear protein. The preparation of nuclei from which the Ca\textsuperscript{2+}-dependent protein kinase is extracted contains no other intact organelles (5). When these nuclei are stripped of their envelopes and of contaminating cytoplasmic membrane fragments by Triton X-100, the 90 kD kinase remains associated with the remaining insoluble fraction, which consists mainly of chromatin, nuclear scaffolding, and nuclear matrix proteins. Also, in an earlier autoradiographic study we showed that when purified nuclei are reacted with \([\gamma-32\text{P}]\text{ATP in the}

Figure 5. Characterization of an antibody (pc475) to a 43 kD phosphoprotein from pea chromatin. A and B, Autoradiograph of SDS-PAGE pattern of \(\text{\textsuperscript{32P}}\)-labeled proteins from pea nuclei phosphorylated in the presence of A, 10 \(\mu\text{M}\) Ca\textsuperscript{2+}; B, 0.1 mm EDTA. C, Western blot analysis of proteins shown in lane F, immunostained by pc475; D, same as C, immunostained by preimmune serum; E, \(\text{\textsuperscript{32P}}\)-labeled protein immunoprecipitated by pc475 from a preparation of chromatin-associated proteins after their extraction from pea nuclei labeled with \([\gamma-32\text{P}]\text{ATP}; F, Coomassie brilliant blue staining pattern of proteins extracted from pea chromatin by 0.3 M NaCl and electrophoresed on SDS-PAGE.

Figure 6. Fluorescence micrographs showing immunocytochemical stain of pea nuclei by preimmune serum (a) and pc475 (b). Cells from formaldehyde-fixed, unembedded pea plumes were teased from the tissue in a droplet of buffer, then broken and spread onto a chrom-gelatin treated slide under light pressure from a glass coverslip. The dispersed cell contents were then dried and immunostained as described in "Materials and Methods." Identity of structures as nuclei was confirmed by Nomarski optics. Many spherical (e.g. at arrow) and oblong nuclei are visible in each sample. The spherical nucleus marked by the arrow in each sample is 10 \(\mu\text{m}\) in diameter.

Copyright © 1991 American Society of Plant Biologists. All rights reserved.
presence of Ca\(^{2+}\), more than 90% of the label is bound within the nuclei (5).

The 90 kD kinase could have become associated with nuclei during their extraction, but at least one of its principal substrates has a nuclear locale in situ. This substrate, whose phosphorylation in purified nuclei is stimulated threefold by 10 \(\mu\)M Ca\(^{2+}\) (Fig. 5), has a \(M_r\) of 43,000. The pc475 antibodies raised to this protein band immunoprecipitate a labeled \(M_r\)

43,000 phosphoryshopeptide from nuclei reacted with \([\gamma-32P]ATP\), and, as assayed immunocytologically, they strongly label nuclei from unembedded cells that were fixed in intact pea plumules (Fig. 6). They also label nuclei in thin sections of fixed and embedded plumule cells (M Dauwalder, C-G Tong, SJ Roux, unpublished data). This finding indicates that the same 43 kD substrate whose phosphorylation is stimulated by 10 \(\mu\)M Ca\(^{2+}\) in isolated nuclei is also present in pea nuclei in situ. One would expect that the Ca\(^{2+}\)-stimulated kinase that phosphorylates this substrate would also have a nuclear locale. However, final proof that it is nuclear will require detecting it there by immunocytochemical methods.

Using different resolving conditions and a different sets of standards, we previously reported that the phosphorylation of a pea nuclear protein near 47 kD could be stimulated by red light and calcium (4). Under our current conditions of extraction and SDS-PAGE analysis of phosphorylated proteins from pea nuclei, the only protein band between 40 and 50 kD whose phosphorylation is enhanced by Ca\(^{2+}\) has a \(M_r\) of 43,000. The phosphorylation of this band may also be regulated by light, and we are currently testing this possibility.

The Ca\(^{2+}\)-dependence and calmodulin independence of the 90 kD kinase raise the question of its possible relationship to PKC and to other Ca\(^{2+}\)-dependent protein kinases. However, the pea kinase does not phosphorylate the myelin basic protein peptide (Table III), which is a strongly favored peptide substrate of PKC (30), and it is relatively unaffected by sphingosine (Table II), which is a potent inhibitor of PKC (9). Also, it is threefold less sensitive than PKC to inhibition by staurosporine (26). The pea kinase also differs from the several other calmodulin- and phospholipid-independent, calcium-dependent protein kinases that have been isolated from plants (8, 20, and references therein): it is considerably larger than the other purified CDPKs and is thus far the only one shown to phosphorylate a nuclear protein.

Because the calmodulin antagonist, chlorpromazine, inhibits both phytochrome-stimulated transcription of the Chl a/b message (12) and phytochrome-stimulated phosphorylation of nuclear proteins (4), Lam et al. speculated that a calmodulin-regulated protein kinase may play a key role in the transduction chain leading from phytochrome activation to changes in gene expression (12). However, chlorpromazine, W\(_7\), and most other calmodulin antagonists are equally effective against most proteins that undergo Ca\(^{2+}\)-dependent conformation changes, whether they are calmodulin dependent or not (8, 10, 18). Thus, inhibition of the 90 kD Ca\(^{2+}\)-dependent protein kinase by chlorpromazine and W\(_7\) could account, in part, for the effects of these inhibitors on light-induced gene expression even though this kinase is calmodulin independent. Further analysis of both the 90 kD protein kinase and its protein substrates in pea nuclei will aid evaluation of this hypothesis.

**ACKNOWLEDGMENT**

We thank C-G. Tong for help in preparing tissue sections from pea stems for immunocytochemical analyses.

**LITERATURE CITED**


