Pea DNA Topoisomerase I Is Phosphorylated and Stimulated by Casein Kinase 2 and Protein Kinase C

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DNA topoisomerase I catalyzes the relaxation of superhelical DNA tension and is vital for DNA metabolism; therefore, it is essential for growth and development of plants. Here, we have studied the phosphorylation-dependent regulation of topoisomerase I from pea (Pisum sativum). The purified enzyme did not show autophosphorylation but was phosphorylated in an Mg²⁺-dependent manner by endogenous protein kinases present in pea nuclear extracts. This phosphorylation was abolished with calf intestinal alkaline phosphatase and lambda phosphatase. It was also phosphorylated by exogenous casein kinase 2 (CK2), protein kinase C (PKC; from animal sources), and an endogenous pea protein, which was purified using a novel phorbol myristate acetate affinity chromatography method. All of these phosphorylations were inhibited by heparin (inhibitor of CK2) and calphostin (inhibitor of PKC), suggesting that pea topoisomerase I is a bona fide substrate for these kinases. Spermine and spermidine had no effect on the CK2-mediated phosphorylation, suggesting that it is polyamine independent. Phospho-amino acid analysis showed that only serine residues were phosphorylated, which was further confirmed using antiphosphoserine antibody. The topoisomerase I activity increased after phosphorylation with exogenous CK2 and PKC. This study shows that these kinases may contribute to the physiological regulation of DNA topoisomerase I activity and overall DNA metabolism in plants.

DNA is very stable in double-stranded form in the genome, but it goes through topological alterations in the course of various cellular functions, including replication, repair, recombination, transcription, nucleosome assembly, and chromosome segregation. DNA topoisomerases are a class of enzymes that catalyze and control the interconversion of topological states of DNA to maintain the superhelical density of DNA and, thus, help in maintaining the genome integrity (see Wang, 1996, 2002; Nitiss, 1998). Based on their mechanisms of action, these enzymes are classified as type I or type II topoisomerases. The type I enzymes are usually monomeric and transiently break one strand of duplex DNA, allowing for single-step changes in the linking number of circular DNAs. Type II enzymes are dimeric and break both the strands of a duplex to generate a gate through which another region of DNA can be passed, resulting in linking number changes in steps of two (see Wang, 1996). Eukaryotic type I topoisomerases do not require ATP, metal cofactors, or single-stranded DNA for their activity; they form a covalent intermediate with the 3' end of the broken strand and are able to relax both positive and negative supercoils (Wang, 1996; Mudgil et al., 2002).

DNA topoisomerase I has been isolated and characterized from bacterial, viral, animal, and yeast systems (Cozzarelli and Wang, 1990). The enzyme from fruitfly (Drosophila melanogaster) was shown to be essential for its growth and development (Lee et al., 1993). Little is known about DNA topoisomerases from plant system. The plant topoisomerase I was purified and characterized from wheat (Triticum aestivum) germ (Dyvan et al., 1981), cultured tobacco (Nicotiana tabacum) cells (Pagliuso et al., 1990), broccoli (Brassica oleracea; Kieber et al., 1992a), and pea (Pisum sativum; Chiatante et al., 1993). There are few reports on the isolation of topoisomerase I genes (top I) from plants such as Arabidopsis (Kieber et al., 1992b), carrot (Daucus carota; Balestrazzi et al., 1996, 2000), and pea (Reddy et al., 1998). Recently, we have reported the cloning and characterization of a cell cycle-regulated top I gene from tobacco, which was induced by light, cold, and abscisic acid (Mudgil et al., 2002). The catalytic activity of topoisomerase I is known to be regulated by posttranslational modifications including phosphorylation/dephosphorylation, and these modifications may play a physiological role during cell growth (Coderoni et al., 1990; D’Arpa and Liu, 1995). The regulation of topoisomerase I activity by phosphorylation and its involvement in DNA-protein interactions may be one of the mechanisms by which protein kinases and phosphatases control gene expression (Coderoni et al., 1990). Phosphorylation by casein kinase 2 (CK2) and protein kinase C (PKC) and dephosphorylation of ani-

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/10.1104/pp.103.024273.
mal topoisomerase I have been shown to enhance or reduce its catalytic activity, respectively (Durban et al., 1983; Pommier et al., 1990; Samuels and Shimizu, 1992). In contrast, it has been reported that phosphorylation by a Tyr protein kinase decreased the catalytic activity of calf thymus DNA topoisomerase I (Tse-Dinh et al., 1984). In plants, the regulation of DNA topoisomerase I by phosphorylation has not been well studied. We have reported previously that tobacco topoisomerase I is a phosphoprotein and a substrate for PKC (Mudgil et al., 2002). We have also reported the cloning and characterization of pea topoisomerase I (Reddy et al., 1998) and its stimulation by a pea DNA helicase 45 (Pham et al., 2000). The purified pea topoisomerase I was shown to relax both positive and negative supercoiled DNA in absence of the divalent cation, Mg$^{2+}$. However, in the presence of Mg$^{2+}$, the pea enzyme was shown to introduce positive supercoils, a unique property not reported in the enzyme from any other organism except archaebacteria (Reddy et al., 1998). In this report, we describe the regulation of pea DNA topoisomerase I by phosphorylation. We show that this enzyme is phosphorylated by endogenous and exogenous CK2 and PKC protein kinases at Ser residue(s), resulting in an increase in its catalytic activity.

RESULTS

Phosphorylation of Pea Topoisomerase I by Endogenous Protein Kinases

Pea nuclear extract (NE) was used as a source of endogenous protein kinases to phosphorylate purified recombinant pea topoisomerase I. The results show that it phosphorylates the 100-kD pea topoisomerase I polypeptide (Fig. 1A, lane 1). No phosphorylation was detected if the enzyme (NE, lane 2) or substrate (pea topoisomerase I, lane 3) were omitted from the phosphorylation reactions (Fig. 1A, lanes 2 and 3). No significant phosphorylation of pea topoisomerase I by NE was observed when Mg$^{2+}$ was omitted (Fig. 1B, lane 1). However, effi-

Figure 1. In vitro phosphorylation of pea DNA topoisomerase I by endogenous kinases and its dephosphorylation. Pea NE (NE, 3 μg) was used as source of endogenous kinases to phosphorylate recombinant pea DNA topoisomerase I (0.5 μg). The standard kinase reaction was carried out in the presence of Mg$^{2+}$ and [γ$^{32}$P]-ATP followed by SDS-PAGE and autoradiography as described in “Materials and Methods.” A, Autoradiogram showing a 100-kD band of pea topoisomerase I phosphorylated with NE (lane 1). Lanes 2 and 3, Reactions with only topoisomerase I (without NE) and NE (without topoisomerase I). B, Autoradiogram showing the phosphorylation of topoisomerase I in absence (lane 1) and presence of 0.5, 1.0, and 2.0 mM MgCl$_2$ (lanes 2–4). C, Autoradiogram showing that there is no autophosphorylation of topoisomerase I at 0.5- and 2.0-μg concentrations (lanes 2 and 3). Lane 1 is a standard kinase reaction. D, Autoradiogram showing topoisomerase I phosphorylation (lane 1) is inhibited by calf intestinal alkaline phosphatase (CIAP) and lambda phosphatase (LP) (lanes 2 and 3). E, Autoradiogram showing the phosphorylation of topoisomerase I (lane 1) is inhibited by 3 μM calphostin (lane 3) and 25 μg of heparin (lane 4) and not inhibited even by higher concentration (100 μM) of stauroscopine (lane 2).
Phosphorylation of Pea Topoisomerase I by Exogenous CK2 and PKC Protein Kinases

To test the phosphorylation by exogenous protein kinases, frog (Xenopus laevis) CK2 and rat (Rattus rattus) PKC were used as a source of exogenous protein kinases to phosphorylate the pea topoisomerase I. The results show that pea topoisomerase I was phosphorylated by CK2 (Fig. 2A, lane 2), whereas topoisomerase I had no autophosphorylation activity (lane 1), and this phosphorylation was inhibited by heparin, an inhibitor of CK2 (Fig. 1E, lanes 3 and 4). However, staurosporine even at high concentrations (100 μM) did not inhibit this phosphorylation (Fig. 1E, lane 2). These results suggest that the endogenous protein kinases involved in this phosphorylation may be CK2 and PKC.

Pea Topoisomerase I Is Phosphorylated at Ser Residue(s)

The CK2 and PKC phosphorylated 100-kD polypeptide bands of pea topoisomerase I were eluted from the gel and subjected to phospho-amino acid analysis followed by paper chromatography. This analysis revealed that phosphorylation occurred on Ser residue(s) of the topoisomerase I by CK2 or PKC (Fig. 3A, lanes 1 and 2). This was further confirmed by antiphospho-Ser antibody. Figure 3B shows the inhibition of CK2 phosphorylation in presence of phospho-Ser antibody (lane 1) as compared with that without the antibody (lane 2). Similar results were also observed with the PKC phosphorylation (data not shown).

One-Step Purification of Pea PKC

To check for an endogenous PKC-like activity in pea NE, we have developed a novel method for its purification by using PMA-Sepharose affinity column chromatography. The PMA was first coupled to epoxy-conjugated Sepharose4B. The pea NE was loaded on this PMA-Sepharose column, and the column was excessively washed with a buffer A containing 100 mM NaCl, followed by elution of bound PKC with 4α-PMA. The kinase active fraction was checked by SDS-PAGE and found to be almost pure (Fig. 4A, lane 2) with molecular mass of 70 kD. After dialysis, this pure pea PKC was used to phosphorylate the pea topoisomerase I. The results showed that pea topoisomerase I was phosphorylated by the purified pea PKC (Fig. 4B, lane 3). As a negative control, no phosphorylation was observed in reactions lacking either PKC or topoisomerase I (Fig. 4B, lanes 1 and 2).

Stimulation of Pea Topoisomerase I Activity by Phosphorylation with CK2 and PKC

We tested the effects of phosphorylation on pea topoisomerase I activity using a DNA relaxation assay. A normal DNA ladder formation is seen with pea topoisomerase I (Fig. 5, A and B, lane 1). This activity was stimulated when topoisomerase I was prephosphorylated with either CK2 (Fig. 5A, lane 3) or PKC (Fig. 5B, lane 3), and as a result, the supercoiled DNA moved up. The CK2 and PKC preparations themselves showed no ability to relax supercoiled DNA (lane 4 in Fig. 5, A and B). This indicated that the stimulation of activity was due to the phosphorylation of topoisomerase I by CK2 and PKC protein kinases.

DISCUSSION

Topological problems arise in almost all the intracellular DNA transactions either due to circularity of DNA or from the extreme length of genomic DNA.
These problems are resolved by using two types of DNA topoisomerases (I and II), which are among the most conserved proteins (Wang, 1996, 2002) and are regulated by posttranslational modifications. The amount and stability of DNA topoisomerase I do not significantly change during the cell cycle (Gorsky et al., 1989). There appears to be general agreement that the DNA relaxation activity of DNA topoisomerase I is regulated by phosphorylation and dephosphorylation (Durban et al., 1983; Kaiserman et al., 1988; Pommier et al., 1990). It has been reported that the enzyme in eukaryotic system is a nuclear phosphoprotein, and its dephosphorylation abolishes the strand relaxation activity (Kaiserman et al., 1988).

DNA topoisomerase I has been isolated from a number of plants, but its regulation has not been well studied. Previously, we have reported the isolation and characterization of pea topoisomerase I (Reddy et al., 1991). In vitro phosphorylation of pea DNA topoisomerase I by exogenous kinases and its inhibition. The *Xenopus laevis* CK2 and rat brain PKC were used as source of exogenous kinases to phosphorylate recombinant pea DNA topoisomerase I (0.5 μg). The standard kinase reaction was carried out in presence of Mg²⁺ and [γ⁻³²P]ATP followed by SDS-PAGE and autoradiography as described in “Materials and Methods.” A, Autoradiogram showing a 100-kDa band of pea topoisomerase I phosphorylated with CK2 (lane 2). The phosphorylation reactions were performed with 1, 5, 10, and 25 μg of heparin (lanes 3–6, respectively). Lane 1, Reaction with only topoisomerase (without CK2). B, Autoradiogram showing phosphorylation of topoisomerase I with PKC in the presence of 5, 10, and 20 μg of phorbol myristate acetate (PMA; lanes 2–4, respectively). This phosphorylation is inhibited by 3 μM calphostin (Cal., lane 1) in presence of 20 μg of PMA. C, Concentration curve of calphostin. The PMA (20 μg)-stimulated phosphorylation of topoisomerase I with PKC in the absence (lane 1) and in the presence of 0.2, 0.5, and 1.0 μM calphostin (lanes 2–4, respectively). D, Autoradiogram showing the phosphorylation of topoisomerase I with CK2 (lane 1) in the presence of 2.5 mM spermine (lane 2) and 2.5 mM spermidine (lane 3).
et al., 1998). Here, we show that the pea DNA topoisomerase I is phosphorylated in vitro by pea NE. This phosphorylation was Mg$^{2+}$/H$_{11001}$ dependent and was inhibited by inhibitors of CK2 (heparin) and PKC (calphostin), thus confirming that these two protein kinases were involved in its phosphorylation. The phosphorylation was not inhibited by staurosporine, a general inhibitor of PKC suggesting that pea topoisomerase I may be a specific substrate of PKC. The pea topoisomerase I does not exhibit autophosphorylation or any other kinase activities. In contrast, the human DNA topoisomerase I was reported to also contain kinase activity, which phosphorylated the pre-mRNA splicing factors (Rossi et al., 1998). In the present study, we further show that the pea topoisomerase I was also phosphorylated by exogenously added CK2 and PKC from animal sources and that this phosphorylation was inhibited by their respective inhibitors. These data further confirm that the pea enzyme is a bona fide substrate for these protein kinases. The CK2 is a multifunctional, cyclic nucleotide- and calcium-independent, Ser/Thr-specific protein kinase and is known to play an important role in a variety of processes, such as mitosis and cell growth, signal transduction, DNA replication, and the transcription and translation of mRNA (Pinna, 1990; Pepperkok et al., 1991; Li and Roux, 1992; Roux, 1993; Hinrichs et al., 1993; Klimczak et al., 1995). X. laevis topoisomerase I was also reported to be a substrate for CK2 (Kaiserman et al., 1988) similar to pea topoisomerase I. In contrast, the tobacco topoisomerase I was not a substrate for CK2 (Mudgil et al., 2002). The physical association of CK2 and X. laevis DNA topoisomerase I also has been reported, which may modulate the catalytic activity of the enzyme (Kordiyak et al., 1994). CK2 is also reported to be present in the nucleus, thus physically placing it in a relevant subcellular location for regulating DNA topoisomerase activity in cells (Li and Roux, 1992). The present study is the first demonstration that plant topoisomerase I is a substrate for CK2. Another biochemical property of CK2 is its activation by polyamine (Pinna, 1990), which has been proposed to be growth regulators in plants (see Roux, 1993). However, in the present study the CK2 phosphorylation of pea topoisomerase I was not affected
by spermine and spermidine, suggesting that this CK2 could be a different class of enzymes that is polyamine independent.

In this study, we have shown that pea topoisomerase I is also phosphorylated by PKC, which is a Ser kinase and is known to be a key enzyme in the signal transduction pathway. Similar to pea topoisomerase I, PKC was also reported to phosphorylate mammalian and tobacco topoisomerase I (Pommier et al., 1990; Mudgil et al., 2002). PKC phosphorylation was also stimulated by PMA. Here, we have also identified a novel PMA-stimulated PKC activity in pea NE. This activity was purified in a single step by using PMA-Sepharose affinity chromatography, a new method developed in this study. The nuclear PMA-kinase used pea topoisomerase I as a substrate. The phosphorylation of pea topoisomerase I by CK2 and PKC was at Ser residue(s). In animal systems, topoisomerase I is also reported to be phosphorylated at Ser residue(s) by both PKC (Pommier et al., 1990) and CK2 (Kordiyak et al., 1994).

We also have shown in this study that the pea topoisomerase I activity was stimulated after phosphorylation by CK2 or PKC. It has been reported earlier that phosphorylation of topoisomerase I on Ser residues by a CK2-like enzyme (Durban et al., 1983; Kaiserman et al., 1988) or by PKC (Pommier et al., 1990; Samuels and Shimizu, 1992) stimulates its catalytic activity. In contrast, phosphorylation at Tyr residues by a protein Tyr kinase has been shown to decrease the catalytic activity of calf thymus DNA topoisomerase I by greater than 90% (Tse-Dinh et al., 1984).

A number of reports suggest a role for DNA topoisomerase I in the regulation of gene expression, and it has been shown to interact preferentially with active genes (Gellert, 1981; Wang, 1996, 2002). It is likely to allow DNA swirling and relaxation of positive DNA supercoiling ahead of active transcription complexes (Wang, 1996, 2002). The regulation of DNA topoisomerase I activity by phosphorylation may be one mechanism through which protein kinases could control gene expression. The results of this study suggest the phosphorylation by CK2 or PKC may contribute to the physiological regulation of DNA topoisomerase I activity in the plant cell. This would increase our understanding of the link between signal transduction and gene expression in plant systems. Furthermore, we have also developed a new method for purifying a novel PMA-stimulated pea PKC in a single chromatographic step. This would help in identification of PKC-like proteins and their substrates in plants.

**MATERIALS AND METHODS**

**Materials and Buffers**

Supercoiled plasmid (pBR322) DNA was prepared as described (Sambrook et al., 1989). \([\gamma^{32}P]ATP\) was purchased from NEN Life Sciences (Boston). Calphostin, heparin, staurosporine, spermine, spermidine, CIAP, lambda phosphatase, PMA, 4a-PMA, phospho-Ser, phospho-Tyr, phospho-Thr, phenylmethanesulfonyl fluoride (PMSF), sodium bisulfite, and electrophoresis M₉ markers were obtained from Sigma (St. Louis). Monoclonal antiphospho-Ser antibody was purchased from Amersham (Buckinghamshire, UK). Epoxy-conjugated Sepharose-4B was from Pharmacia Corp. (Uppsala). CK2 α-subunit cDNA clone from *Xenopus laevis* was a gift from Prof. Jorge E. Allende and Catherine C. Allende (University of Chile, Santiago, Chile). This clone was expressed in bacteria, and the CK2 (alpha) protein was purified to homogeneity as described (Hinrichs et al., 1993). PKC from rat brain was purchased from Promega (Madison, WI).

The following buffers were used: NE-1 buffer, 0.55 M Suc, 50 mM Tris-Cl (pH 8.0), 10 mM MgCl₂, 25 mM KCl, 10 mM Na₂SO₄, 7 mM β-mercaptoethanol, and 0.5 mM PMSF; and NE-2 buffer, 600 mM KCl, 50 mM Tris-Cl (pH 7.9), 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 25% (v/v) glycerol, 0.5 μM leupeptin, 0.5 mM PMSF, and 1 mM pepstatin. Buffer A was 20 mM HEPEs (pH 8.0), 50 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, 1 mM PMSF, and 1 mM sodium bisulfite.

**Phosphorylation of Pea Topoisomerase I**

The open reading frame (2,676 bp) of pea cDNA clone (3,055 bp) was further subcloned in bacterial vector pET28a to overexpress and purify the encoded protein (Reddy et al., 1998). The protein was present in inclusion body and purified through column chromatography on S-Sepharose (Pharmacia), heparin Sepharose (Pharmacia), and nickel-nitrilotriacetic acid agarose (Qiagen USA, Valencia, CA) columns as described (Reddy et al., 1998). The purified pea DNA topoisomerase I was homogeneously pure with a molecular mass of 100 kDa.

**Preparation of NE**

The pea NE was prepared from the top three to four leaves of 7- to 8-d-grown pea seedlings as described (Tuteja et al., 2001). In brief, the leaves were washed with ice-cold phosphate-buffered saline, submerged in ice-cold NE-1 buffer, and homogenized in a kitchen mixer. Thereafter, the homogenate was passed through two layers of cheesecloth and two layers of Miracloth (Calbiochem, San Diego). The filtrate was then centrifuged at 1,000×g for 10 min at 4°C in a Sorvall RC 5B centrifuge (Kendro Products, Newtown, CT). The pellet was slowly resuspended in NE-1 buffer containing 2.5% (v/v) Triton X-100 and incubated at 4°C with slow shaking to lyse the chloroplasts. This was followed by centrifugation at 2,000×g for 30 min at 4°C. If the pellet was still green in color, the above step was repeated until all the chloroplasts were removed. The resulting nuclear pellet was then resuspended in NE-2 buffer and homogenized in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000×g for 30 min at 4°C, and the clear supernatant was dialyzed against buffer containing 50 mM KCl, 50 mM Tris-Cl (pH 8.0), 20% (v/v) glycerol, and protease inhibitors and stored at −80°C. This preparation was used as the NE.

**Protein Kinase Assays**

In vitro phosphorylation of pea DNA topoisomerase I was performed with PKC and CK2 and endogenous kinases from pea NE. For the PKC assay, 130 μL of the reaction mixture contained 30 mM HEPEs (pH 7.5), 10 mM MgCl₂, 2 mM CaCl₂, 5 mM EGTA, 4 μg of phosphatidyl-Ser, 10 mM PMA, 500 ng of purified pea topoisomerase I, and 5 units of rat brain PKC. The reaction was initiated by adding \([\gamma^{32}P]ATP\) to a final concentration of 1 μM (2 μCi) and incubated at 30°C for 10 min. The reaction was terminated by adding 15 μL of 4% Laemmli SDS sample buffer. This was loaded on a SDS-10% (w/v) polyacrylamide gel, fractionated by electrophoresis, and analyzed by autoradiography. For the CK2 assay, the reaction was performed as for PKC, but CaCl₂, PMA, phosphatidyl-Ser, and EGTA were omitted, and *X. laevis* CK2 (2 μg) was used instead of PCK as the phosphorylating kinase. The phosphorylation of pea topoisomerase I by endogenous
protein kinases from pea NE was performed as described for CK2 except that NE (3 μg) was used instead of CK2.

**Dephosphorylation Reaction**

Pea topoisomerase I was phosphorylated by NE as described above and run on SDS-polyacrylamide gel. The phosphorylated 100-kD isotopically labeled protein band was cut from the gel, and the protein was eluted. This fraction was treated with CIAP or lambda phosphatase in a reaction containing 50 mm Tris-HCl (pH 8.5), 1 mm MgCl₂, and 0.1 mm ZnCl₂ at 30°C for 15 min., followed by SDS-PAGE, and analyzed by autoradiography.

**Phospho-amino Acid Analysis**

The phosphorylated topoisomerase I band was eluted from the gel and hydrolyzed in 6 N HCl for 2 h at 105°C as described (Hunter and Setton, 1980). After hydrolysis, the sample was concentrated in a Speed Vac (Thermo Savant, Holbrook, NY) and analyzed by paper chromatography on Whatman 3MM paper (Whatman, Clifton, NJ). Along with this sample, the standard samples (phospho-Ser, phospho-Tyr, and phospho-Thr) were also run and analyzed as described (Mudgil et al., 2002).

**DNA Topoisomerase I Assay**

Pea DNA topoisomerase I activity was quantitated by agarose gel electrophoresis to monitor the relaxation of supercoiled (form I) pBR322 plasmid DNA in a total volume of 40 μL. The reaction was incubated at 30°C for 10 min. and terminated by the addition of 0.5% (v/v) SDS. After digestion with 50 ng ml⁻¹ proteinase K for 15 min at 56°C, bromphenol blue-γlycerol (0.05%:10% [w/v]) was added and the reaction products were analyzed by electrophoresis in 1% (w/v) agarose gel at 2 V cm⁻¹ for 6 h in 40 mm Tris base, 20 mm acetic acid, and 2 mM Na₂-EDTA buffer (pH 8.1). Topoisomers were visualized by ethidium bromide staining. For stimulation of pea topoisomerase I activity by protein kinases, the enzyme was prephosphorylated with CK2 or PKC before performing the topoisomerase I assay.

**Purification of PKC-Like Protein from Pea Nuclei Using PMA Affinity Chromatography**

For preparation of the affinity column, PMA was first covalently coupled to epoxy-conjugated Sepharose 4B using the supplier’s protocol (Pharmacia). For purification of PKC, the pea NE was passed through the PMA-affinity column. The flow through was recycled once to increase the binding efficiency. The column was washed with 10 column volumes of buffer A followed by buffer A containing 100 mm NaCl. The bound protein (PKC) was eluted from the affinity column with 5 column volumes of a linear gradient from 100 to 50 μg ml⁻¹ 4α-PMA in buffer A. The kinase active fractions were found to be approximately 90% pure as checked on SDS-PAGE and were pooled and used in the phosphorylation assay.

**ACKNOWLEDGMENTS**

We thank Prof. Jorge E. Allende and Catherine C. Allende (University of Chile, Santiago) for a gift of CK2 cDNA clone of alpha subunit from X. laevis, Dr. Renu Tuteja and Dr. Shahid Jameel (International Centre for Genetic Engineering and Biotechnology, New Delhi, India) for critical reading of the manuscript, and Mr. Tran-Quang Ngoc (International Centre for Genetic Engineering and Biotechnology, New Delhi, India) for his help in the preparation of the illustrations.

Received March 26, 2003; returned for revision April 23, 2003; accepted May 4, 2003.

**LITERATURE CITED**


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Tuteja N, Beven AF, Shaw PJ, Tuteja R (2001) A pea homologue of human DNA helicase I is localized within the dense fibrillar component of the nucleolus and stimulated by phosphorylation with CK2 and cdc2 protein kinases. Plant J 25: 9–17