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Characterization of a Calcium-Dependent Protein Kinase from *Arachis hypogea* (Groundnut) Seeds[1]

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A calcium-dependent protein serine/threonine kinase (GnCDPK) has been detected in groundnut (*Arachis hypogea*) seeds that specifically phosphorylates a peptide (MLCpep) representing the phosphate-accepting domain of smooth muscle myosin light chains. GnCDPK has been purified to near homogeneity from the soluble fraction of groundnut seeds by ammonium sulfate precipitation, Q Sepharose, Blue Sepharose, and Sephacryl 300 chromatography. The molecular weight of GnCDPK is estimated to be 53,000. Enzyme activity is stimulated about 100-fold in the presence of free Ca^{2+} (concentration required for half-maximal activation = 0.5 μM). GnCDPK is capable of binding $^{45}Ca^{2+}$ ions directly in an electroblot, indicating it to be a calcium-binding protein. Phosphorylation of MLCpep is found to be optimal at an alkaline pH range (pH 9-10). Unlike all other calcium-dependent protein kinases reported from higher plants, GnCDPK does not accept casein or histones as substrate. Sequences related to MLCpep (>60% homologous) that are present in myosin light chains from skeletal muscles of chicken and rabbit also fail to act as a substrate for GnCDPK. In contrast to the Ca^{2+}/calmodulin dependence of myosin light chain kinases, GnCDPK activity is not affected by the presence of exogenous calmodulin (1-10 μM). However, enzyme activity is considerably inhibited in the presence of calmodulin antagonists like *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (concentration required for 50% inhibition [IC_{50}] = 30 μM) and calmidazolium (IC_{50} = 10 μM), indicating an endogenous calmodulin structure to be present in GnCDPK. The probability of GnCDPK being a bona fide plant myosin light chain kinase is discussed.

CDPKs act as sensors for intracellular calcium flux and translate them into physiological responses by reversibly phosphorylating Ser and/or Thr residues of relevant key enzymes in different biochemical pathways (Edelman et al., 1987). CDPKs are found to be of two different kinds: (a) Ca^{2+}/calmodulin dependent (i.e. protein kinases that are activated by Ca^{2+} only in the presence of calmodulin; Stull et al., 1986); and (b) Ca^{2+}/phospholipid dependent (i.e. protein kinases that need phospholipid along with Ca^{2+} for their activation; Nishizuka, 1988). Several reports have demonstrated the presence of both kinds of protein kinases in plants (reviewed by Hetherington et al., 1990). In addition, plants have been shown to contain another group of CDPKs that are independent of both calmodulin and phospholipids (Harmon et al., 1987; Battey and Venis, 1988; Putnam-Evans et al., 1990). A well-characterized soybean CDPK from this group has been found to contain a calmodulin-like regulatory domain (Harper et al., 1991).

Ca^{2+} ions have been implicated as a second messenger in important physiological processes in plants, e.g. their response to light, gravity, growth regulators, stress factors, etc. (Hepler and Wayne, 1985; Trewavas, 1986). Ca^{2+}-dependent phosphorylations are also well documented in plants (Poovaiah and Reddy, 1987; Ranjeva and Boudet, 1987; Roberts and Harmon, 1992). Similar to those in animal systems, these phosphorylations are turning out to be a major biochemical mechanism in plant signal transduction.

Characterization of protein kinases involved in such phosphorylation reactions is a prerequisite for understanding the relevant biochemical cascade(s). This report describes the identification, purification, and characterization of a CDPK from groundnut (*Arachis hypogea*, commonly known as peanut) seeds. The enzyme activity does not appear to be regulated by exogenous calmodulin, although calmodulin antagonists like W7 and calmidazolium inhibit the enzyme activity. GnCDPK has been found to be highly selective for the peptide MLCpep as its exogenous substrate. MLCpep represents the minimal sequence necessary for recognition and phosphorylation of SmMLCs by MLCKs in a Ca^{2+}/calmodulin-dependent manner.

MATERIALS AND METHODS

Materials

Groundnut (*Arachis hypogea*) seeds of the JL 24 variety were obtained from the National Research Centre for Groundnuts (Gujrat, India). MLCpep was obtained from Peninsula Laboratories (Belmont, CA, catalog number 8654). [γ-^{32}P]ATP (specific activity 4000 Ci/mmol), [γ-^{32}P]GTP (specific activity 3000 Ci/mmol), and $^{45}CaCl_2$ (specific activity 72 mCi/g) were obtained from Bhaba Atomic Research Center (Bombay, India). Q Sepharose and Sephacryl 300 were from

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Abbreviations: CDPK, calcium-dependent protein kinase; GnCDPK, groundnut calcium-dependent protein kinase; IC_{50}, concentration required for 50% inhibition; $K_{0.5}$, concentration required for half-maximal activation; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCpep, synthetic peptide representing the myosin light chain kinase phosphorylation site of smooth muscle myosin light chains; SkMLC, skeletal muscle myosin light chain; SmMLC, smooth muscle myosin chain; W5, *N*-(6-aminohexyl)-1-naphthalene sulfonamide; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide.

Pharmacia. Blue Sepharose was prepared according to Botime et al. (1972). W7, W5, calmidazolium, MLCs from rabbit skeletal muscle, histone type IIIS, histone type IIS, dephosphorylated casein, phosvitin, and the cellulose TLC plates were obtained from Sigma. $CaCl_2$ and Mg acetate were obtained from BDH Chemical Co. (Dagenham, Essex, UK). All other reagents of analytical grade were obtained from Qualigens (Bombay, India).

Methods

Protein Kinase Assay

The rate of incorporation of ^{32}P into the synthetic peptide substrate MLCpep (KKRPQRATSNVFS) corresponding to the phosphorylation site of smooth muscle myosin-P light chain was measured. A standard protein kinase assay was done according to DasGupta et al. (1989) with the following modifications: (a) reaction buffer was Tris-HCl, pH 7.4; (b) the reaction was initiated by the addition of 100 μM [γ-^{32}P]ATP (300 cpm/pmol); and (c) substrate peptide concentration was 100 μM. For autophosphorylation of GnCDPK, the same conditions were used except (a) exogenous substrate was not added; (b) the reaction was initiated by the addition of 1 μM [γ-^{32}P]ATP (3000 cpm/pmol); and (c) incubations were done for 5 min and the reaction was terminated by the addition of SDS-PAGE sample buffer. For Ca^{2+}-dependence curves, free Ca^{2+} levels were set using Ca^{2+}/EGTA buffers as described by Martell and Smith (1974).

Preparation of Proteins

Chicken gizzard muscle MLCs and SkMLCs were prepared according to Blumenthal and Stull (1980). All other protein preparations were made as described by DasGupta et al. (1989).

Purification of GnCDPK

All steps were performed at 4°C unless mentioned otherwise. Groundnut seeds (200 g) were dry ground at room temperature using a regular kitchen grinder. The powder was stored at −70°C overnight. The frozen cake of groundnut powder was brought to room temperature and homogenized in 400 mL of buffer A (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 0.1% β-mercaptoethanol), with 10 μg/mL leupeptin. The crude homogenate (450 mL) was centrifuged at 15,000 rpm for 30 min. Crude supernatant (285 mL) thus obtained was centrifuged at 100,000*g* for 1 h to obtain the soluble supernatant (170 mL). Soluble supernatant was subjected to 25 to 45% ammonium sulfate fractionation. The pellet was dissolved in 120 mL of buffer A containing 10 μg/mL leupeptin and was dialyzed against 5 L of buffer A overnight. The dialysate was centrifuged for 10 min at 10,000*g* to remove the aggregated insoluble proteins. The supernatant thus obtained was applied to a 40-mL Q Sepharose column equilibrated with buffer A at a flow rate of 1 mL/min. The column was washed with about 1 L of buffer A containing 0.1 M NaCl until the flow was free of absorbance at 280 nm.

GnCDPK was eluted from the column using a 400-mL linear gradient of 0.1 to 0.4 M NaCl in buffer A. The active fractions (3 mL/fraction) were pooled and dialyzed against buffer A. The Q Sepharose fraction was supplemented with 2.5 mM $MgCl_2$ and 200 μM $CaCl_2$ and loaded on a 10-mL Blue Sepharose column equilibrated with buffer A containing 2.5 mM $MgCl_2$ and 200 μM $CaCl_2$. GnCDPK activity was eluted from this column using a 200-mL linear gradient of 0 to 0.6 M NaCl in buffer A. Active fractions (5 mL/fraction) were pooled and concentrated in Amicon 30,000 cut-off cones. The concentrated protein (3 mL) was subjected to gel filtration in a Sephacryl 300 (90 × 2 cm) column equilibrated with buffer A containing 0.2 M NaCl. Elution of GnCDPK was carried out at a flow rate of 30 mL/h using the same buffer. Active fractions (2 mL/fraction) were pooled, concentrated using Amicon 30,000 cut-off cones, and stored at −20°C.

Protein Determination

Protein concentrations were determined by the method of Bradford (1976). BSA was used as standard.

Phosphoamino Acid Analysis

MLCpep was phosphorylated by pure GnCDPK in standard protein kinase assay conditions as outlined above except that (a) the specific activity of ATP was 3000 cpm/pmol, and (b) the concentration of MLCpep was 200 μM. The reaction mixture was analyzed in 17.5% SDS-PAGE according to Laemmli (1970). The wet gel was autoradiographed (2-h exposure) and the position of the phosphorylated peptide was identified. The corresponding gel was cut, crushed using a mortar and pestle, and left overnight in 1:1 (v/v) MilliQ-purified (Millipore, Marlborough, MA) H_2O. The eluted peptide was dried and subjected to hydrolysis in 6 N HCl for 3 h. Hydrolysates were mixed with standard P-Ser and P-Thr and analyzed by thin-layer electrophoresis on cellulose plates at pH 1.9 (88% formic acid:glacial acetic acid:H_2O, 50:156:1794) at 1500 V as described by Cooper et al. (1983). Radiolabeled amino acids were identified by autoradiography and the standards were identified by ninhydrin staining.

RESULTS

Detection of GnCDPK

A significant level of CDPK activity (1 nmol min^{-1} mg^{-1} protein) was detected in the soluble fraction of groundnut seeds by using the peptide KKRPQRATSNVFS (MLCpep) as substrate. Analysis of a standard protein kinase reaction mixture (see "Materials and Methods") in SDS-PAGE showed that the incorporated radioactivity was due to phosphorylation of the peptide (Fig. 1, lane a). Endogenous phosphorylation events were not detectable under the assay conditions used. MLCpep phosphorylation was completely inhibited in the presence of EGTA (Fig. 1, lane c), indicating it to be a Ca^{2+}-dependent phenomenon. Control reaction mixtures without the peptide were analyzed in lanes b and d. The absence of any radioactive band in these lanes rules out the possibility of endogenous peptide phosphorylation events.

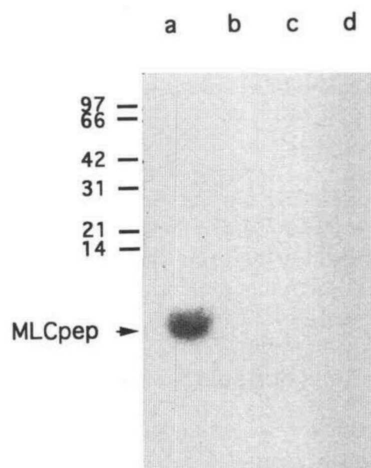

Figure 1. SDS-PAGE analysis of MLCpep phosphorylation by GnCDPK. Standard protein kinase assay reaction mixtures were electrophoresed in 17.5% gels according to Laemmli (1970). Two micrograms of protein from a soluble supernatant fraction of groundnut seeds was used as the source of GnCDPK activity. Lane a, Standard reaction mixture; lane b, standard reaction mixture without MLCpep; lane c, standard reaction mixture with 1 mM EGTA; lane d, standard reaction mixture without MLCpep and with 1 mM EGTA. The figure is an autoradiogram. The arrow indicates the position of MLCpep in the gel.

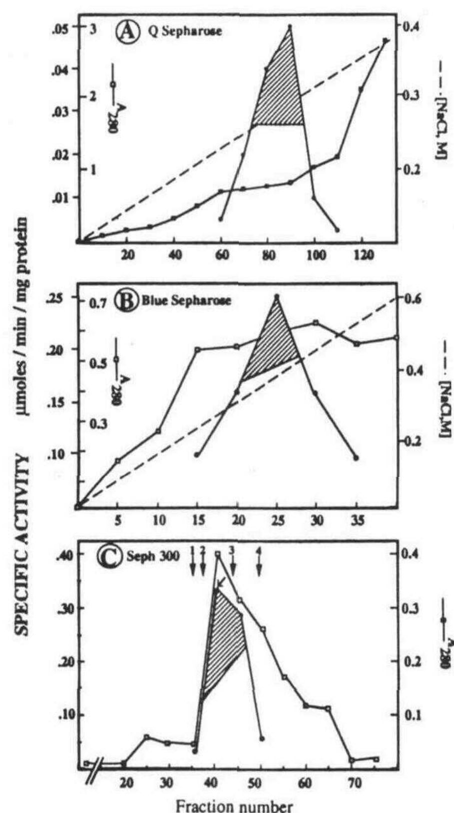

Figure 2. GnCDPK elution profiles for chromatography on different columns. Details of the procedures are described in "Materials and Methods." The dotted lines indicate the progress of the salt gradients used for GnCDPK elutions. The shaded areas indicate the fractions that were pooled and processed/stored. A, Q Sepharose chromatography. B, Blue Sepharose chromatography. C, Sephacryl 300 chromatography. The arrows indicate the elution positions of the markers used for calibrating the gel filtration column: 1, phosphorylase b, 97,000; 2, BSA, 67,000; 3, ovalbumin, 43,000; 4, carbonic anhydrase, 31,000. The unnumbered arrow indicates the elution position of GnCDPK.

Purification of GnCDPK

GnCDPK was detected and characterized from a single batch of freshly harvested groundnut seeds. The basal GnCDPK activity in the crude extracts from the seeds did not show any appreciable variation (tested every 30 d) over the 1-year period of its dormancy (M. DasGupta, unpublished observations). The standard purification of GnCDPK was about 350-fold (as shown in Table I) with an approximate yield of 9%.

Elution profiles of the three major chromatographic steps used for the purification of GnCDPK are shown in Figure 2. The 25 to 50% ammonium sulfate fraction of the soluble 100,000*g* supernatant was subjected to anion-exchange chromatography using Q Sepharose. The enzyme activity eluted as a single peak at about 0.25 M NaCl from this column (Fig. 2A). GnCDPK activity was Ca^{2+} dependent in the Q Sepharose fractions, but addition of 1 to 10 µM exogenous calmodulin did not have any additional effect. The absence of free calmodulin in the Q Sepharose eluant was verified by the eluant's inability to substitute for calmodulin in a Ca^{2+}/calmodulin-dependent standard MLCK assay performed according to DasGupta et al. (1989). Calmodulin eluted at about 0.5 to 0.6 M NaCl from Q Sepharose (data not shown). These observations indicate that GnCDPK is not dependent on calmodulin for its activity. However, the possibility of GnCDPK having calmodulin as a subunit could not be ruled out at this point.

Table I. *Purification of GnCDPK from groundnut seeds*

Fraction	Volume	Protein	Specific Activity		Purification	Total Activity	Yield
			$+Ca^{2+}$	$-Ca^{2+}$			
	mL	*mg*	*nmol min⁻¹ mg⁻¹*		*fold*		%
Soluble supernatant	170	3910	1.0	0.06	1	3910	100
Ammonium sulfate	120	1000	2.1	0.09	2.1	2100	53.7
Q Sepharose	80	50	39	1.7	39	1950	49.8
Blue Sepharose	30	6	243	2.5	243	1458	37.2
Sephacryl 300	15	1	351	3.6	351	351	8.9

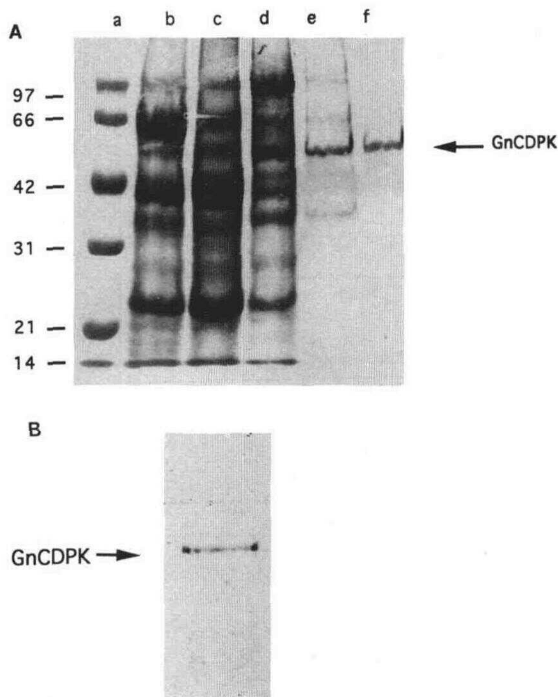

Figure 3. A, SDS-PAGE analysis of the course of purification of GnCDPK. Proteins from different purification pools were resolved in a 10% polyacrylamide gel in the presence of SDS and stained with Coomassie blue. Lane a, Molecular mass markers in kD; lane b, soluble supernatant (25 μg); lane c, 25 to 45% ammonium sulfate fraction (25 μg); lane d, Q Sepharose fraction (15 μg); lane e, blue Sepharose fraction (3 μg); lane f, Sephacryl 300 pool (2 μg). B, GnCDPK analysis by electrophoresis in nondenaturing conditions. The Sephacryl 300-pooled fraction (2 μg) was analyzed in a 7.5% polyacrylamide gel according to Laemmli (1970) in the absence of SDS (nondenaturing condition). The arrow indicates the position of GnCDPK.

The Q Sepharose eluant was subjected to cibacron blue Sepharose chromatography in the presence of Ca^{2+} and Mg^{2+}. From this column, GnCDPK was eluted using a 0- to 0.6-M NaCl gradient in the presence of EDTA (Fig. 2B). Most of the contaminants of GnCDPK were removed by this step as shown by electrophoretic analysis in Figure 3A, lane e. Final purification was obtained by gel filtration through a Sephacryl 300 column (Fig. 2C). Figure 3A shows the Coomassie blue staining pattern of the SDS-PAGE analysis of the course of purification. The active fractions (2 μg) from the gel filtration column showed a single band, under both nondenaturing (Fig. 3B) and denaturing conditions (Fig. 3A, lane f). To check the purity further, silver staining was done for 10 μg of the pure GnCDPK preparation (data not shown). The pattern that emerged indicated a single major band at the same position as GnCDPK, with three very minor bands (<1%) at the 48-, 36-, and 22-kD positions. One possible explanation for the appearance of such bands is proteolytic processing of the protein or, alternatively, a minor population of related proteins could have co-purified. Purification results thus indicate GnCDPK to be a single subunit protein, ruling out the possibility of calmodulin being a subunit of GnCDPK.

There was no effect of exogenous calmodulin on pure GnCDPK activity, which confirms that the enzyme is Ca^{2+} dependent but calmodulin independent.

Estimation of Mol Wt

The mol wt of GnCDPK was estimated to be 53,000 by gel filtration in a Sephacryl 300 column. (Fig. 2C). SDS-PAGE analysis also indicates the same mol wt for GnCDPK (Fig. 3A, lane f). The enzyme thus appears to be active in its monomeric form.

Calcium and Magnesium Ion Dependence

GnCDPK activity was stimulated about 100-fold by the presence of the Ca^{2+} ion (Table I). Half-maximal activation of the enzyme was observed at a free Ca^{2+} concentration of 0.5 μM (Fig. 4A). The optimum concentration of Mg^{2+} needed

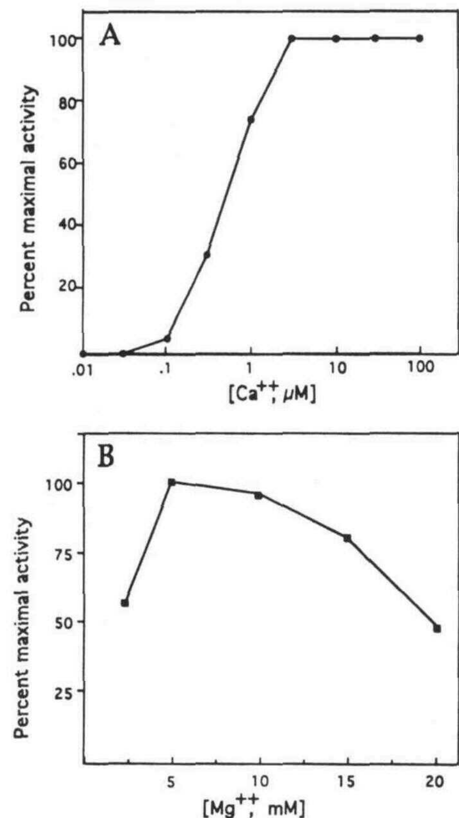

Figure 4. Effect of Ca^{2+} and Mg^{2+} ion concentrations on GnCDPK activity. A, Effect of Ca^{2+}. Activity of GnCDPK (50 ng) was monitored in a standard protein kinase assay mixture at different concentrations of free calcium by using Ca^{2+}/EGTA buffers. Catalytic rates are expressed relative to the reaction rate in standard assay conditions. The data are an average of two determinations done in duplicate. B, The effect of Mg^{2+}. GnCDPK activity was monitored in standard protein kinase assay mixture at different concentrations of Mg^{2+}. Catalytic rates are expressed relative to the reaction rate in standard assay conditions. The data are an average of two determinations done in duplicate.

for the kinase activity was found to be 5 to 10 mM at saturating concentrations of Ca^{2+} (Fig. 4B).

Substrate Specificity

Pure GnCDPK activity was found to be highly specific for the sequence of MLCpep as its exogenous substrate. The K_m and V_{max} values for MLCpep were found to be 50 μM and 0.4 μmol min^{-1} mg^{-1} protein, respectively, from the Lineweaver-Burk plot shown in Figure 5A. The intact MLC corresponding to MLCpep, obtained from chicken gizzards, was also accepted as substrate by GnCDPK, with comparable kinetic parameters. The K_m for the intact chain was found to be 150 μM and the V_{max} was 0.1 μmol min^{-1} mg^{-1} protein. GnCDPK activity was tested with exogenous substrates like histone IIIS, histone IIS, phosvitin, casein, and BSA. With these substrates very low or no activity was noted, as described in Table II. Interestingly, MLCs from skeletal muscles of chicken and rabbit also could not act as substrates for GnCDPK (Table II). Alignment of the sequences of the phosphate-accepting domains of various MLCs (Fig. 6) indicates greater than 60% homology existing in a length of 15 amino acid residues. The data indicate the importance of the nonhomologous residues (unblocked residues, Fig. 6) present in MLCpep in determining its specificity in acting as a substrate for GnCDPK.

Mg^{2+}/ATP was used as a phosphate donor in the GnCDPK-catalyzed reactions. The K_m and V_{max} were found to be 10 μM and 0.4 μmol min^{-1} mg^{-1} protein, respectively, from the Lineweaver-Burk plots (Fig. 5B). Incorporation of phosphate into MLCpep was also detectable in the presence of [γ-^{32}P]GTP. The estimated K_m was found to be 200 μM (20 times lower than that observed with Mg^{2+}/ATP) and the V_{max} was 7 nmol min^{-1} mg^{-1} protein, which is about 60-fold lower than that observed in the presence of ATP. A net 1000-fold lower V_{max}/K_m ratio for GTP compared with ATP makes the role of GTP as phosphate donor less likely in GnCDPK-catalyzed reactions.

Table II. *Substrate specificity of GnCDPK*

Protein kinase assays were done under standard conditions with 50 ng of pure GnCDPK preparation in the presence of the indicated amounts of test substrates. Catalytic rates are expressed relative to the reaction rate obtained in the presence of MLCpep.

Substrate Added	Concentration	Percent Maximal Activity
MLC pep	100 μM	100
Histone IIIS	2 mg/mL	<2
Histone IIS	2 mg/mL	<2
Phosvitin	2 mg/mL	0
Casein	2 mg/mL	0
BSA	2 mg/mL	0
Chicken SmMLC	2 mg/mL	50
Chicken SkMLC	2 mg/mL	0
Rabbit SkMLC	2 mg/mL	0

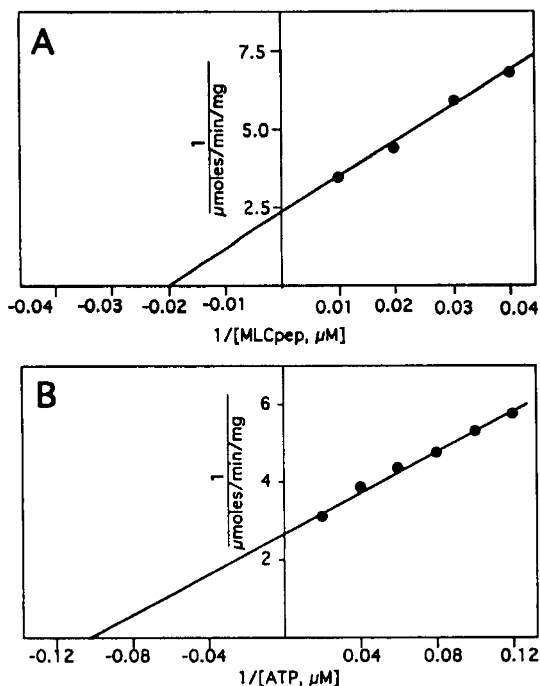

Figure 5. Determination of K_m and V_{max} of MLCpep and Mg^{2+}/ATP for GnCDPK. Protein kinase assays were performed in standard assay conditions with 50 ng of pure GnCDPK preparation and different concentrations of ATP or MLCpep substrate. A, The plot of 1/*v* versus 1/[Mg^{2+}/ATP]; B, the plot of 1/*v* versus 1/[MLCpep].

Effect of pH and Salt

GnCDPK exhibited maximal activity in the alkaline pH range. The phosphorylating activity of the enzyme increases exponentially from pH 5.5 to 9.0 and levels off at pH 9.0 to 10.2 (Fig. 7A). High pH optima have been observed in the case of some histone kinases from both animal and plant systems (Rubin and Rosen, 1975; Lin and Key, 1980). In view of the fact that Arg residues have high pK_a values, it is possible that the ionic status of an Arg residue(s) is a critical determinant for successful GnCDPK-MLCpep interaction. GnCDPK activity has been found to be quite stable in the presence of high salt concentration. Only 25% loss of activity was noted in the presence of 500 mM NaCl (Fig. 7B). Such stability in the presence of salt indicates that GnCDPK-MLCpep interaction is not simply ionic in nature.

Effect of Calmodulin and Calmodulin Antagonists

The activity of GnCDPK and its sensitivity toward Ca^{2+} do not appear to be regulated by exogenous calmodulin (1–10 μM). Interestingly, however, calmodulin antagonists had con-

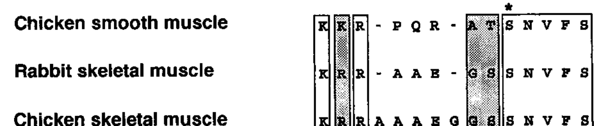

Figure 6. Comparison of the phosphorylation site sequence of various MLCs. The Ser residue that accepts the phosphate group is indicated by a star. Homologous residues are enclosed in boxes, and conservative substitutions are shaded.

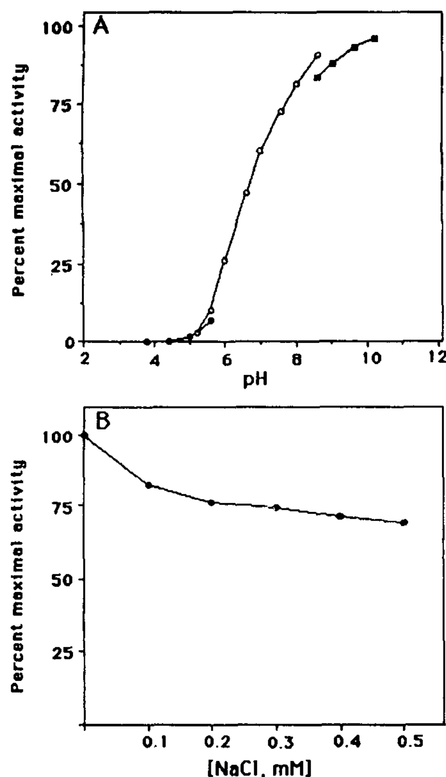

Figure 7. Effect of pH and salt on GnCDPK activity. Protein kinase assays were performed in standard assay conditions with 50 ng of pure GnCDPK preparation. A, The effect of pH. The following buffers were used at 50 mM concentrations at indicated pH values. ●, Na acetate buffers; ○, Tris malate buffers; ■, Gly-NaOH buffers. Catalytic rates are expressed relative to the reaction rate in 50 mM Gly-NaOH buffer, pH 10.2, in otherwise standard conditions. The data are averages of two determinations done in duplicate. B, The effect of increasing concentrations of NaCl. Catalytic rates are expressed relative to the reaction rate in standard assay conditions. The data are averages of two determinations done in duplicate.

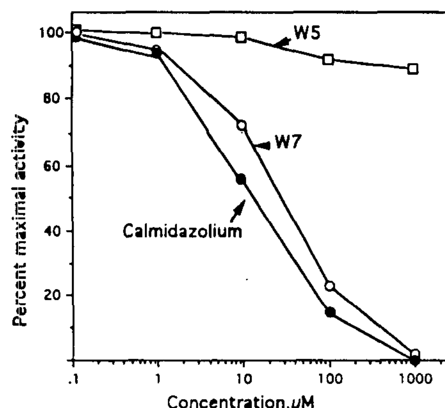

Figure 8. Effect of various calmodulin antagonists on pure GnCDPK activity. Protein kinase assays were performed in standard assay conditions with 50 ng of pure enzyme preparation and indicated amounts of inhibitors. The buffer/enzyme mixture was incubated for 5 min with the inhibitor before addition of the peptide substrate. Catalytic rates are expressed relative to the reaction rate in the absence of any inhibitor in standard conditions.

siderable inhibitory effect on GnCDPK activity. W7 inhibited with an IC_{50} of 30 μM, but W5, the less potent analog, had no inhibitory effect. Calmidazolium, another potent calmodulin antagonist, also inhibited GnCDPK at an IC_{50} of 10 μM (Fig. 8). These observations suggest that GnCDPK activity is supported by a calmodulin-like activity that is impaired in the presence of these antagonists.

Effect of Polyamines and Heparin

Polycations like spermine, spermidine, and putrescine at 1 mM concentration did not have any effect on GnCDPK activity (Table III), but a polyanion like heparin completely inhibited the activity of GnCDPK at 200 μM concentration. This inhibition is possibly a substrate-directed property of GnCDPK, because heparin does not have any effect on autophosphorylation of the enzyme (M. DasGupta, unpublished observation). A probable mode of action of heparin inhibition is the masking of the positive charge of a basic residue(s) present in MLCpep that may be crucial for enzyme substrate interaction.

Direct Binding of Calcium by GnCDPK

Most of the calcium-binding proteins show a mobility shift in SDS-PAGE in the presence of Ca^{2+}. GnCDPK failed to show any such shift in mobility (data not shown), but when GnCDPK was electroblotted and incubated with $^{45}Ca^{2+}$ a strong signal was obtained following autoradiography (Fig. 9). This clearly shows that GnCDPK is capable of binding calcium directly. There are other Ca^{2+}-binding proteins, like calcemedins, that do not show Ca^{2+}-dependent mobility shifts in SDS-PAGE. It should also be noted that only one of the two bands of soybean CDPK showed a Ca^{2+}-dependent mobility shift (Harmon et al., 1987).

Phosphoamino Acid Analysis of Phosphorylated MLCpep

The peptide substrate of GnCDPK has three possible sites of phosphorylation, namely Ser^{9}, Ser^{13}, and Thr^{8}. The amino acid residues phosphorylated by GnCDPK were identified by thin-layer electrophoresis of hydrolyzed samples of MLCpep. GnCDPK catalyzes the incorporation of phosphate mainly in Ser (99%) (Fig. 10). The rest is incorporated in Thr. Which of

Table III. *Effect of polyamines and heparin on GnCDPK activity*

Protein kinase assays were done in standard conditions with 50 ng of pure GnCDPK preparation in the presence of the indicated effectors. Catalytic rates are expressed relative to the reaction rate in the absence of any effector in standard conditions.

Addition	Concentration	Percent Maximal Activity	
		$+Ca^{2+}$	$-Ca^{2+}$
Spermine	1 mM	100	1
Spermidine	1 mM	100	1
Putrescine	1 mM	100	1
Heparin	200 μM	5	0.8

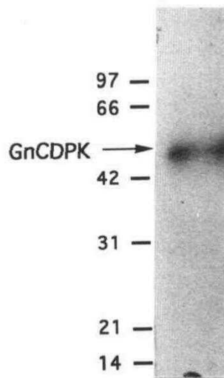

Figure 9. Binding of $^{45}Ca^{2+}$ to electroblotted GnCDPK. Pure GnCDPK was electrophoresed in a 10% gel and then electroblotted in Immobilon membranes (Millipore). The blot was incubated with $^{45}CaCl_2$ (300 μCi/10 mL) as described by Maryyama et al. (1984).

the two Ser residues is being phosphorylated cannot be said at present, but Ser^9 seems to be the likely candidate because this Ser is specifically phosphorylated by MLCKs in animal systems (Kemp et al., 1983). In fact, SmMLC kinase phosphorylates MLCpep in similar Ser:Thr 97:3 ratios.

Western Blot Analysis of GnCDPK with Monoclonal Antibodies Directed against Soybean CDPK

Monoclonal antibodies specific against a CDPK from suspension-cultured soybean cells (Putnam-Evans et al., 1990) were used to examine the immunocross-reactivity of GnCDPK with soybean CDPK. As shown in Figure 11, cross-reaction was observed with the GnCDPK preparation in the position of GnCDPK (indicated by the arrow), suggesting similarities in the primary structure of the two kinases. Although not clearly visible in the figure, there were two more very faint bands at the 48- and 36-kD positions (positions indicated by short arrows) that cross-reacted with soybean CDPK antibodies. Interestingly, both of these faint bands correspond to the minor contaminants found in pure GnCDPK preparation (described earlier). Results indicate that

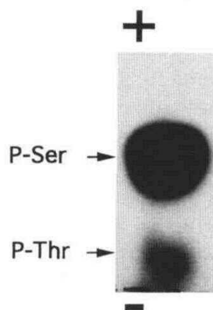

Figure 10. Identification of phosphorylated amino acid(s) in MLCpep. Details of the procedure are given in "Materials and Methods." Phosphorylated MLCpep was hydrolyzed in 6 N HCl. Hydrolysate was dried, dissolved in water, and subjected to thin-layer electrophoresis at pH 1.9. The positions of the standards visualized by ninhydrin staining are indicated by arrows.

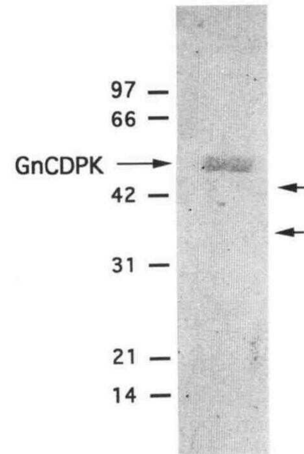

Figure 11. Western blot analysis of GnCDPK. GnCDPK (2 μg from blue Sepharose fraction) was analyzed by 10% SDS-PAGE, electroblotted to Immobilon membranes, and immunostained with a mixture of monoclonal antibodies specific for a soybean CDPK according to Putnam-Evans et al. (1990). (Immunostaining was kindly done by Dr. Alice Harmon.)

these minor bands are related to GnCDPK, and a possible and most likely explanation would be that they are proteolytic fragments of GnCDPK.

Autophosphorylation of GnCDPK

Purified GnCDPK was found to autophosphorylate itself when incubated with [γ-^{32}P]ATP and Mg^{2+} (Fig. 12, lane b). Similar to exogenous substrate phosphorylation, GnCDPK autophosphorylation was also inhibited in the presence of

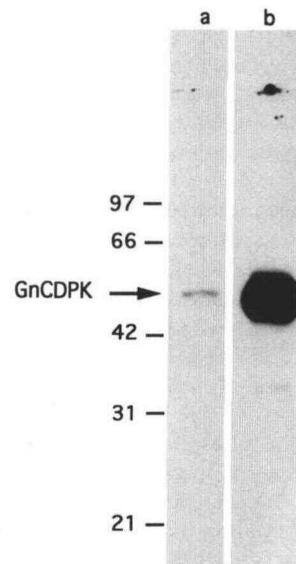

Figure 12. Autophosphorylation of GnCDPK. Purified GnCDPK (5 μg) was autophosphorylated as described in "Materials and Methods." Reaction mixtures were analyzed by 10% SDS-PAGE, and the dried gel was autoradiographed. Lane a, Standard reaction mixture with 1 mM EGTA; lane b, standard reaction mixture.

EGTA (Fig. 12, lane a). This indicates that autophosphorylation of GnCDPK is a Ca^{2+}-dependent phenomenon.

DISCUSSION

The results in this report describe the purification and characterization of a CDPK from groundnut seeds. This enzyme, GnCDPK, differs from all other CDPKs reported from higher plants, as indicated by its high selectivity for the SmMLC sequence as an exogenous substrate. Unlike the other reported CDPKs from plants, GnCDPK cannot phosphorylate histones or casein. The data presented here show that sequences homologous (>60% homogeneity) to MLCpep present in SkMLCs are not acceptable as exogenous substrate by GnCDPK. The nonhomologous amino acids present in MLCpep (see Fig. 6) thus seem to be crucial determinants of its unique structure, for which GnCDPK is highly selective. As shown in Figure 6, these nonhomologous amino acids are not spread over the length of the phosphate-accepting domain of the MLCs. Rather, it is a nonhomologous pocket that is flanked by two homologous domains. The phosphate-accepting Ser is located between the nonhomologous pocket and the C-terminal homologous domain. Among the nonhomologous residues, Pro (residue 4) has the unique property of imposing structural rigidity in peptide structures. It is possible that such a structural restraint gives MLCpep the structure that is needed for its recognition by GnCDPK. The other nonhomologous residues are Gln and Arg (residues 5 and 6) in SmMLCs, which are replaced by Glu and Ala residues in corresponding positions of SkMLCs. The resultant basic nature of the nonhomologous pocket of SmMLCs can also act as a potential determinant of its specific interaction with GnCDPK. A CDPK with similar substrate specificity has been identified from a lower plant; *Mougeotia* (Roberts, 1989). With *Mougeotia* CDPK, it was shown that deletion of one of the two basic residues (Gln^5) in the nonhomologous pocket renders SmMLC peptide inactive as a substrate. This is consistent with the conclusions derived from the data presented here. It should also be noted that the importance of basic residues in the GnCDPK-MLCpep interaction was also indicated by the heparin inhibition of the enzyme activity (Table III).

Recently, it has been demonstrated that the smooth and skeletal muscle MLCKs from animals can be distinguished by their different substrate specificities (Leachman et al., 1992). Skeletal muscle MLCK phosphorylate MLCs isolated from skeletal or smooth muscles with similar efficiency. In contrast, the smooth muscle MLCK specifically phosphorylates SmMLCs. This is exactly what is found to be the substrate selection property of GnCDPK. It accepts SmMLCs but does not recognize and/or phosphorylate SkMLCs. Moreover, both GnCDPK and smooth muscle MLCK phosphorylate Ser and Thr residues in the MLCpep sequence. The mechanism of specific substrate binding by the skeletal muscle MLCKs and smooth muscle MLCKs is due to subtle differences in the structure of their catalytic cores (Herring et al., 1992). The catalytic core of GnCDPK thus has a high probability of being similar to the smooth muscle MLCK. Is GnCDPK then actually a plant MLCK? It is worth mentioning here that two CDPKs, one from soybean and another from the alga *Chara*, have been found to be co-localized with F-actin in plant cells by immunocross-reactivity studies. This observation indicated the enzymes to be associated with the contractile system (Putnam-Evans et al., 1989; McCurdy and Harmon, 1992). Interestingly, monoclonal antibodies against soybean CDPK have been found to cross-react with GnCDPK, indicating the similarity between these two proteins (Fig. 11). Although these observations suggest that GnCDPK is a plant MLCK, the conclusive proof will require in vitro biochemical experimentation.

A critical difference in smooth muscle MLCK and GnCDPK concerns their dependence on calmodulin. Smooth muscle MLCK activity is essentially Ca^{2+}/calmodulin dependent, but GnCDPK activity has been found to be unaffected by exogenous calmodulin. However, GnCDPK activity has been found to be inhibited in the presence of calmodulin antagonists. This antagonism can only be directed toward an endogenous calmodulin-like structure present in GnCDPK because (a) calmodulin is not present as a contaminant in the pure enzyme preparation and (b) calmodulin is not a subunit of GnCDPK (Fig. 3, A and B). It is possible that the inhibition caused by the calmodulin antagonists are directed toward a calmodulin-like regulatory domain integrated in GnCDPK's primary structure, as is described in soybean CDPK (Harper et al., 1991). The fact that the Ca^{2+}-dependent regulation of GnCDPK appears to be through direct binding of the divalent ion to the enzyme (Fig. 9) is consistent with the prediction of GnCDPK having an endogenous calmodulin-like domain.

It was interesting to find out that GnCDPK has several biochemical properties in common with the CDPK characterized from soybean (Harmon et al., 1987; Putnam-Evans et al., 1990). The similarities are: (a) The mol wts are almost the same, 52,000 and 55,000 for soybean CDPK and 53,000 for GnCDPK. (b) Enzyme activities in both cases are stimulated by Ca^{2+}. The $K_{0.5}$ for Ca^{2+} activation is 2.1 μM for soybean CDPK, which is only 4 times higher than that obtained for GnCDPK (0.5 μM). (c) Both enzymes can bind Ca^{2+} ions directly. (d) Like GnCDPK, one of the two soybean CDPK bands does not show Ca^{2+}-dependent mobility shift in SDS-PAGE. (e) Kinase activities in both cases are inhibited by calmodulin antagonists. (f) Neither of them are stimulated by exogenous calmodulin. (g) Both are Ser/Thr kinases. (h) Both the enzymes can undergo Ca^{2+}-dependent autophosphorylation. These similarities are consistent with the noted immunocross-reactivity between the two enzymes.

The important differences that indicate the nonidentity between these enzymes are: (a) Substrate specificity: GnCDPK is highly specific for SmMLC sequence as its exogenous substrate. Soybean CDPK phosphorylated histone IIIS along with the SmMLC sequence. (b) Phosphate donor selection: soybean CDPK can utilize both Mg^{2+} GTP and Mg^{2+}/ATP at comparable efficiencies, whereas GnCDPK is very specific for Mg^{2+} ATP. (c) Sensitivity to polycations: although soybean CDPK was inhibited in the presence of polycations, GnCDPK activity was not affected by their presence at all. (d) pH dependence: soybean CDPK showed the usual bell-shaped pH-dependence curve in a range of pH 6.0 to 10.0 with histone IIIS as substrate. Phosphorylation of MLCpep by GnCDPK, however, reached a plateau at alkaline pH range. Autophosphorylation of GnCDPK, however, showed

a bell-shaped pH-dependence curve similar to soybean CDPK (M. DasGupta, unpublished observations), indicating the plateau activity at alkaline pH to be an exogenous substrate-directed property of GnCDPK. It would be useful to know the pH dependence of soybean CDPK activities with Sm-MLCs as substrate as well as that of its autophosphorylation for further comparative analysis regarding this point. Taken together, the above comparison suggests that soybean CDPK and GnCDPK are highly related nonidentical proteins. Finally, it should be noted that GnCDPK is the first protein kinase to be detected and characterized from ungerminated dry seeds.

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