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Dual Regulation of a Chimeric Plant Serine/Threonine Kinase by Calcium and Calcium/Calmodulin*

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A chimeric Ca^{2+}/calmodulin-dependent protein kinase (CCaMK) gene characterized by a catalytic domain, a calmodulin-binding domain, and a neural visinin-like Ca^{2+}-binding domain was recently cloned from plants (Patil, S., Takezawa, D., and Poovaiah, B. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 4797–4801). The *Escherichia coli*-expressed CCaMK phosphorylates various protein and peptide substrates in a Ca^{2+}/calmodulin-dependent manner. The calmodulin-binding region of CCaMK has similarity to the calmodulin-binding region of the α-subunit of multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). CCaMK exhibits basal autophosphorylation at the threonine residue(s) (0.098 mol of ^{32}P/mol) that is stimulated 3.4-fold by Ca^{2+} (0.339 mol of ^{32}P/mol), while calmodulin inhibits Ca^{2+}-stimulated autophosphorylation to the basal level. A deletion mutant lacking the visinin-like domain did not show Ca^{2+}-stimulated autophosphorylation activity but retained Ca^{2+}/calmodulin-dependent protein kinase activity at a reduced level. Ca^{2+}-dependent mobility shift assays using *E. coli*-expressed protein from residues 358–520 revealed that Ca^{2+} binds to the visinin-like domain. Studies with site-directed mutants of the visinin-like domain indicated that EF-hands II and III are crucial for Ca^{2+}-induced conformational changes in the visinin-like domain. Autophosphorylation of CCaMK increases Ca^{2+}/calmodulin-dependent protein kinase activity by about 5-fold, whereas it did not affect its Ca^{2+}-independent activity. This report provides evidence for the existence of a protein kinase in plants that is modulated by Ca^{2+} and Ca^{2+}/calmodulin. The presence of a visinin-like Ca^{2+}-binding domain in CCaMK adds an additional Ca^{2+}-sensing mechanism not previously known to exist in the Ca^{2+}/calmodulin-mediated signaling cascade in plants.

The signal-induced change in free Ca^{2+} concentration in the cytoplasm has been portrayed as a switch that turns on various cellular processes in plants and animals (1–3). Ca^{2+}-mediated protein phosphorylation is one of the major mechanisms by which eukaryotic cells transduce extracellular signals into intracellular responses (4–6). Ca^{2+}/calmodulin-dependent protein kinases are involved in amplifying and diversifying the action of Ca^{2+}-mediated signals (7, 8). In animals, several types of Ca^{2+}/calmodulin-dependent protein kinases have been identified, including myosin light chain kinases, phosphorylase kinase, and EF-2 kinase, as well as Ca^{2+}/calmodulin-dependent protein kinase I, II, and IV (9, 10). The multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMKII)[1] is one of the well characterized kinases, and it is known to play a pivotal role in cellular regulation because of its ability to phosphorylate a large number of proteins (11).

Although Ca^{2+}-dependent protein kinases are found in many plant species (12, 13), little is known about Ca^{2+}/calmodulin-dependent protein kinases in plants. Ca^{2+} and Ca^{2+}/calmodulin-dependent protein phosphorylation has been demonstrated in a number of plant extracts (14–16). However, convincing biochemical evidence for the presence of calmodulin-dependent protein kinase in plants has not been reported previously. Most of the evidence of calmodulin dependence has been indirect, based on the use of calmodulin antagonists and on activation studies with exogenous calmodulin (1, 14, 16). Watillon *et al.* (17) reported a homolog of mammalian CaMKII from plants, but the biochemical properties of this kinase are not known.

CCaMK is a novel Ca^{2+}/calmodulin-dependent protein kinase characterized by two distinct regulatory domains; a visinin-like domain is regulated by Ca^{2+}, while the other is regulated by Ca^{2+}/calmodulin. The visinin-like domain of CCaMK contains three conserved Ca^{2+}-binding EF-hand motifs, similar to neural visinin-like proteins (18, 19), which are members of a family of Ca^{2+}-sensitive regulators. The chimeric feature of CCaMK with three distinct domains in a single polypeptide suggests that it has evolved from a fusion of two genes that are functionally different in origin. The CCaMK gene is preferentially expressed during anther development, and it is regulated in a stage-specific manner during microsporogenesis, which implies that it may play a central role in the development of the male gametophyte (20).

Here we report the biochemical properties of CCaMK, which has structural features resembling both mammalian Ca^{2+}/calmodulin-dependent protein kinases and plant Ca^{2+}-dependent protein kinases. The results presented here show a dual mode of regulation of CCaMK by Ca^{2+} and Ca^{2+}/calmodulin.

EXPERIMENTAL PROCEDURES

Materials—Proteinase inhibitors, histone IIAS, IIIS, myelin basic protein, syntide-2, GS peptide (PLSRTLSVAAKK), myelin basic protein peptide (QKRPSQRSKYL), and spinach calmodulin were purchased from Sigma. [γ-^{32}P]ATP was obtained from DuPont NEN. Calmodulin-Sepharose 4B and Klenow enzyme were obtained from Pharmacia Biotech Inc. Restriction enzymes and biotinylated calmodulin were from Life Technologies, Inc.

Expression and Purification of CCaMK—Escherichia coli cells carrying plasmid pET3b (Novagen, Inc.) containing CCaMK cDNA were induced by isopropyl-1-thio-β-D-galactopyranoside as described earlier (20). Isopropyl-1-thio-β-D-galactopyranoside-induced *E. coli* cells were harvested and suspended in a homogenization buffer (40 mM Tris-HCl,

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[1] The abbreviations used are: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

pH 7.6, 1 mM DTT, 2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of leupeptin, pepstatin, and antipain). Cells were broken by freeze thawing followed by sonication. Subsequent procedures were carried out at 4 °C. The cell extract was clarified by centrifugation at 12,000 × *g* for 30 min. Solid ammonium sulfate (50% saturation) was added to the supernatant and incubated on ice for 1–4 h. The enzyme was recovered by centrifugation for 30 min at 12,000 × *g*. The pellet was solubilized in the column buffer (40 mM Tris-HCl, pH 7.6, 1 mM $CaCl_2$, 1 mM DTT, 10% ethylene glycol, 0.05% Tween 20, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin, pepstatin, and antipain) and applied onto a calmodulin-Sepharose column, which was previously equilibrated with the column buffer. The column was washed first with the column buffer and then with the column buffer containing 1 M NaCl. CCaMK was eluted from the column with buffer containing 40 mM Tris, pH 7.6, 1.5 mM EGTA, 10% ethylene glycol, 0.05% Tween 20, 200 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. Fractions containing the CCaMK were pooled and thoroughly dialyzed against buffer containing 40 mM Tris, pH 7.6, 1 mM DTT, 1 mM EDTA, and 10% ethylene glycol.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (21). Nondenaturing gel electrophoresis was performed using 14% separating gel in 375 mM Tris-Cl, pH 8.8, 5% stacking gel in 125 mM Tris-Cl, pH 6.8, and 25 mM Tris, 192 mM glycine electrophoresis buffer, pH 8.3, at 80 V for 8 h. Protein bands were visualized by staining with Coomassie Brilliant Blue.

Calmodulin-binding Assays—Potato calmodulin PCM6 cDNA (22) was cloned into the pET3b expression vector, and ^{35}S-labeled calmodulin was prepared as described by Fromm and Chua (23). Wild-type and mutant CCaMK proteins were electrophoretically transferred onto nitrocellulose filters and incubated in binding buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 1% (w/v) nonfat dry milk) containing ^{35}S-calmodulin (0.5×10^6 cpm/μg) plus either 1 mM $CaCl_2$ or 5 mM EGTA as described previously (20). Binding assays using biotinylated calmodulin were performed as described previously by Reddy *et al.* (24).

Peptide-binding Assay to Calmodulin—Synthetic peptides were prepared using Applied Biosystems peptide synthesizer 431A in the Laboratory of Bioanalysis and Biotechnology, Washington State University. Different lengths of synthetic peptides were incubated with 100 pmol (1.7 μg) of calmodulin in 10 μl of 20 mM Hepes, pH 7.5, for 5 min and analyzed by nondenaturing polyacrylamide gel electrophoresis.

Deletion Mutants of CCaMK—The mutant construct 1–356 was created by removing a 0.9-kilobase pair *Bam*HI fragment containing the visinin-like domain from the original CCaMK expression plasmid pNY10. The mutant construct 1–322 was created by introducing a *Bgl*II site using an oligonucleotide 5′-GCATTGAAAGATCTCAGTCTAGAAAC-3′. The construct was then inserted into the pET14b expression vector. The mutant proteins were expressed in *E. coli* and purified using either calmodulin-Sepharose column (Pharmacia) or Ni^{2+}-resin column from Novagen, Inc. and the protocol provided by the manufacturer.

Site-directed Mutagenesis and Expression of the Visinin-like Domain—A 0.9-kilobase pair *Bam*HI fragment containing the visinin-like domain of CCaMK was subcloned into M13mp18 RF, and the site-directed mutagenesis was performed (25). Oligonucleotide primers used for the site-directed mutagenesis were 5′-CTCTCATGGCTATAGTTCC-3′ for EF-hand I mutation, 5′-CCTCCTTGGCGATACATCC-3′ for EF-hand II mutation, and 5′-GTCGAACGCGACAACTCC-3′for EF-hand III mutation. An *Nde*I site was created at the position of amino acid residue 358 (Met) using 5′-GGATCCCATCATATGAAATCG-3′. Wild-type and the mutant constructs were then inserted into the pET14b expression vector. All mutant sequences were confirmed by DNA sequencing using the fmol PCR sequencing kit (Promega).

Protein Kinase Assay—Phosphorylation assays (25 μl) were carried out at 30 °C in 50 mM Hepes, pH 7.5, 1 mM DTT, 10 mM magnesium acetate, 200 μM [γ-^{32}P]ATP (1,500–2,000 cpm/pmol) in the presence of either 2.5 mM EGTA or indicated amounts of Ca^{2+} and calmodulin. Protein (0.2 mg/ml) and synthetic peptides (100 μM) were added in the reaction mixture to study substrate phosphorylation. When protein substrates were used, the reaction was terminated by adding SDS-PAGE sample buffer (21) and analyzed on 12% SDS-polyacrylamide gels. Proteins were visualized by staining with Coomassie Brilliant Blue. The gels were dried and subjected to autoradiography. Incorporation of ^{32}P into the substrate was determined by counting the excised protein bands in a liquid scintillation counter. When peptide substrates were used, the reaction was terminated by spotting the reaction mixture on P81 phosphocellulose filters (Whatman). The filters were washed in 75 mM phosphoric acid, and ^{32}P incorporation was determined (26).

Autophosphorylation Assay—The autophosphorylation assay was carried out at 30 °C in the presence of 50 mM Hepes, pH 7.5, containing 10 mM magnesium acetate, 1 mM DTT, 1 mM [γ-^{32}P]ATP (300–400 cpm/pmol), and either EGTA (2.5 mM), $CaCl_2$ (0.5 mM), or $CaCl_2$ (0.5 mM) plus calmodulin (1 μM). For time course assays (100 μl), 1.2 μg (21.4 pmol) of CCaMK and 1 mM [γ-^{32}P]ATP (2,000–3,000 cpm/pmol) were used. Aliquots (10 μl) were transferred at indicated time points into SDS-PAGE sample buffer to stop the reaction. Aliquots for the zero time point were taken immediately after the addition of CCaMK. The samples were then analyzed by 12% SDS-polyacrylamide gel. The amount of phosphate transferred to the enzyme was determined by counting the radioactivity of the excised CCaMK bands in a liquid scintillation counter.

Phosphoamino Acid Analysis—The purified CCaMK (200 ng) was autophosphorylated in the presence of EGTA (2.5 mM) or $CaCl_2$ (0.5 mM) or $CaCl_2$ (0.5 mM) plus 1 μM calmodulin and subjected to SDS-PAGE. The gel was briefly stained with Coomassie Brilliant Blue, CCaMK bands were excised, and the protein was eluted from the gel. The eluted protein was hydrolyzed with 6 N HCl for 2 h at 110 °C and subjected to paper chromatography using propionic acid, 1 M NH_4OH, isopropyl alcohol (45:17.5:17.5) as a solvent (27). Phosphoserine and phosphothreonine standards (5 mg/ml in 10% (w/v) isopropyl alcohol) were visualized by ninhydrin reagent.

RESULTS

To study the Ca^{2+}/calmodulin-dependent kinase activity of CCaMK, the *E. coli*-expressed protein was purified. The protein was essentially pure as revealed by SDS-PAGE and was stable at 4 °C for a few days. The purified protein was used to phosphorylate different substrates such as casein, histones, myelin basic protein, and synthetic peptides. Histone IIAS was found to be the most reactive protein substrate for CCaMK and was used for studying calmodulin concentration-dependent protein kinase activity. The addition of increasing amounts of calmodulin in the presence of 0.5 mM Ca^{2+} stimulated CCaMK activity (Fig. 1*A*). Kinase activity was saturated at calmodulin concentrations around 1.0 μM. The concentration of calmodulin required for half-maximal activity (K_a) of CCaMK was approximately 0.2 μM. The time course studies revealed that histone IIAS phosphorylation was saturated after 10 min in the presence of Ca^{2+}/calmodulin (Fig. 1*B*). In the presence of 2.5 mM EGTA or 0.5 mM Ca^{2+} alone, the enzyme has basal activity that is 10–15-fold lower than the maximal activity achieved with Ca^{2+}/calmodulin. Among other protein substrates tested, CCaMK phosphorylated histone IIIS and myelin basic protein, but it did not phosphorylate phosvitin, phosphoenolpyruvate carboxylase, synapsin I, and casein. CCaMK also phosphorylated synthetic peptides such as GS peptide, myelin basic protein peptide, and syntide-2. Among these peptides, GS peptide was most efficiently phosphorylated by CCaMK in the presence of Ca^{2+}/calmodulin.

Calmodulin-binding affinity of the CCaMK was studied by using different concentrations of ^{35}S-labeled calmodulin. Binding of calmodulin to CCaMK saturated at concentrations above 300 nM (Fig. 2). From the saturation curve, the dissociation constant (K_d) of calmodulin for CCaMK was estimated to be around 55 nM. The binding of calmodulin to CCaMK was completely blocked in the presence of 5 mM EGTA. The Scatchard analysis indicated that CCaMK has a single calmodulin binding site. (Fig. 2, *inset*).

To identify the calmodulin-binding region of CCaMK, truncated mutant constructs were prepared (Fig. 3*A*). The CCaMK mutant 1–356 lacks the COOH-terminal domain, which has high homology to visinin-like proteins. Another CCaMK mutant, 1–322, is further truncated, but it has all 11 domains conserved in serine/threonine protein kinases (28). Wild-type CCaMK (1–520), and truncated mutants 1–356 and 1–322 were expressed in *E. coli* and purified as described under "Experimental Procedures." These proteins were used for ^{35}S-calmodulin binding assays in the presence of Ca^{2+}. The binding of calmodulin to wild-type and mutant 1–356 CCaMKs were sim-

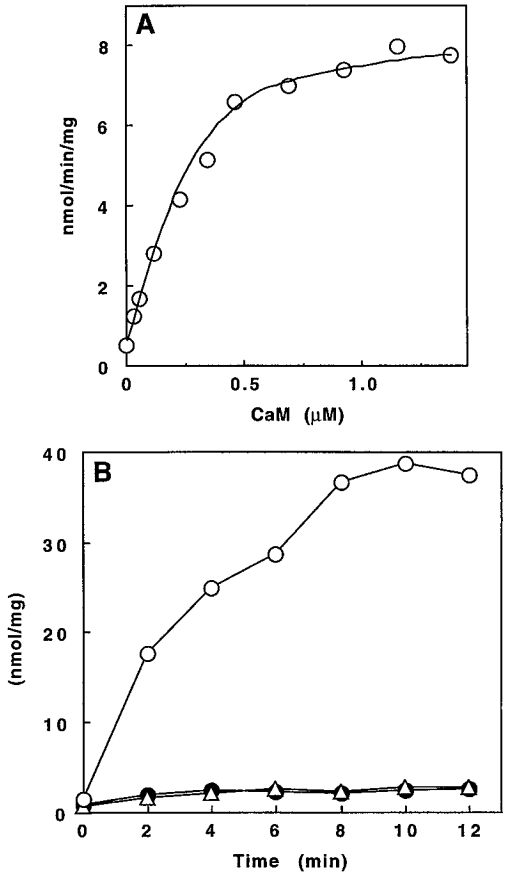

FIG. 1. **Ca^{2+}/calmodulin-dependent protein kinase activity of CCaMK.** *A*, histone IIAS was phosphorylated with CCaMK in the presence of 0.5 mM $CaCl_2$ and increasing amounts of calmodulin (μM) at 30 °C for 2 min. CCaMK activity is presented as nmol of phosphate/min/mg of CCaMK. *B*, time course of phosphorylation of histone IIAS by CCaMK in the presence of 2.5 mM EGTA (●) or 0.5 mM $CaCl_2$ (△) or 0.5 mM $CaCl_2$ and 1 μM calmodulin (○). CCaMK activity is represented as nmol of phosphate/mg of CCaMK.

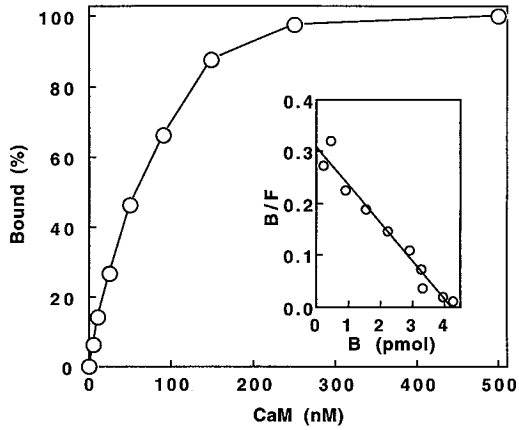

FIG. 2. **Saturation curve of ^{35}S-calmodulin binding to purified CCaMK.** *E. coli*-expressed CCaMK protein (4 pmol) was separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose filter and incubated with different amounts of ^{35}S-labeled calmodulin. After washing in the buffer without ^{35}S-calmodulin, radioactivity of the filter was measured by using a liquid scintillation counter. The amount of bound calmodulin at each point was represented as percent of the maximal binding. The *inset* shows a Scatchard plot of data indicating that the binding ratio of calmodulin to CCaMK is 1:1. Bound/free and bound calmodulin are expressed as *B*/*F* and *B*, respectively.

ilar; whereas, calmodulin did not bind to the mutant CCaMK 1–322 (Fig. 3*A*, *boxed region*), indicating that amino acid residues 322–356 (Fig. 3*A*) are essential for calmodulin-binding to CCaMK. Another mutant CCaMK 1–341 also binds to calmodulin in the presence of Ca^{2+} (data not shown). Similar results were obtained when biotinylated calmodulin was used instead of ^{35}S-calmodulin. Calmodulin binding to wild-type and mutant CCaMKs was prevented by the addition of 5 mM EGTA, indicating the requirement of Ca^{2+} for calmodulin binding. Comparison of amino acid residues of this region of CCaMK corresponding to regions of animal CaMKIIα revealed high homology (Fig. 3*B*).

Different lengths of synthetic peptides from the calmodulin-binding region (amino acid residues 311–340) were used to identify amino acid residues necessary for calmodulin binding. Calmodulin binding to these peptides was studied by gel mobility shift assay using nondenaturing polyacrylamide gel. Calmodulin mixed with peptides 311–340, 317–340, and 322–340 migrated above the position of calmodulin alone; whereas, peptide 328–340 did not affect the mobility of calmodulin (Fig. 3*C*, *top*), suggesting that the calmodulin-binding site exists between amino acid residues 322–340. The addition of these peptides to calmodulin in the presence of 2.5 mM EGTA did not affect the mobility of calmodulin, suggesting that peptide binding to calmodulin is Ca^{2+}-dependent. Increasing amounts of the peptide 322–340 facilitates the gel mobility shift toward the upper position (Fig. 3*C*, *bottom*). Similar results were obtained when the peptides 317–340 and 311–340 were used, suggesting that the amino acid residues 322–340 have a pivotal role in calmodulin binding of CCaMK. The helical wheel projection revealed that amino acid residues 325–338 of CCaMK form a basic amphiphilic α-helix (29) similar to CaMKIIα (Fig. 3*D*).

To study autophosphorylation, CCaMK was incubated at 30 °C with 10 mM magnesium acetate, 1 mM [γ-^{32}P]ATP, and 2.5 mM EGTA. In 30 min, approximately 0.098 mol of ^{32}P/mol of CCaMK was incorporated. This basal autophosphorylation was induced to approximately 3.4-fold in the presence of 0.5 mM $CaCl_2$ (0.339 mol of ^{32}P/mol of CCaMK) (Fig. 4*A*). Increasing the incubation time to 60 min did not improve the stoichiometry of Ca^{2+}-dependent autophosphorylation. Ca^{2+}-dependent autophosphorylation was inhibited to the basal level (0.061 mol of ^{32}P/mol of CCaMK) by the addition of 1 μM calmodulin (Fig. 4*A*). Calmodulin inhibits Ca^{2+}-stimulated autophosphorylation in a concentration-dependent manner (Fig. 4*B*). These results indicate that Ca^{2+} and calmodulin have opposing effects on autophosphorylation of CCaMK. Phosphoamino acid analysis revealed that CCaMK autophosphorylates at the threonine residue(s) (Fig. 4*C*), which was stimulated by Ca^{2+} and inhibited by Ca^{2+}/calmodulin.

Apart from the calmodulin-binding domain, CCaMK has another regulatory domain toward the COOH terminus, which has high homology to animal visinin-like proteins. The visinin-like domain of CCaMK contains three EF-hand motifs with conserved Ca^{2+}-ligating amino acid residues (Fig. 5*A*). To study Ca^{2+}-binding properties of the visinin-like domain of CCaMK, recombinant visinin-like domain protein was expressed in *E. coli*, using the pET14b expression vector. The visinin-like domain protein was expressed to high levels upon induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and most of the protein was present in the soluble fraction. The expressed protein was purified using the Ni^{2+} resin column. The protein eluted from the column with 1 M imidazole buffer was dialyzed in 50 mM Tris-Cl, pH 7.5, and used for Ca^{2+}-dependent mobility shift assay. Electrophoretic mobility of the recombinant visinin-like domain protein was just above the 20.1-kDa molecular weight marker in the presence of 2.5 mM EGTA; whereas, the addition of Ca^{2+} shifted the electrophoretic mobility toward the lower molecular weight (Fig. 5*B*). This suggests that Ca^{2+} binding to the recombinant visinin-

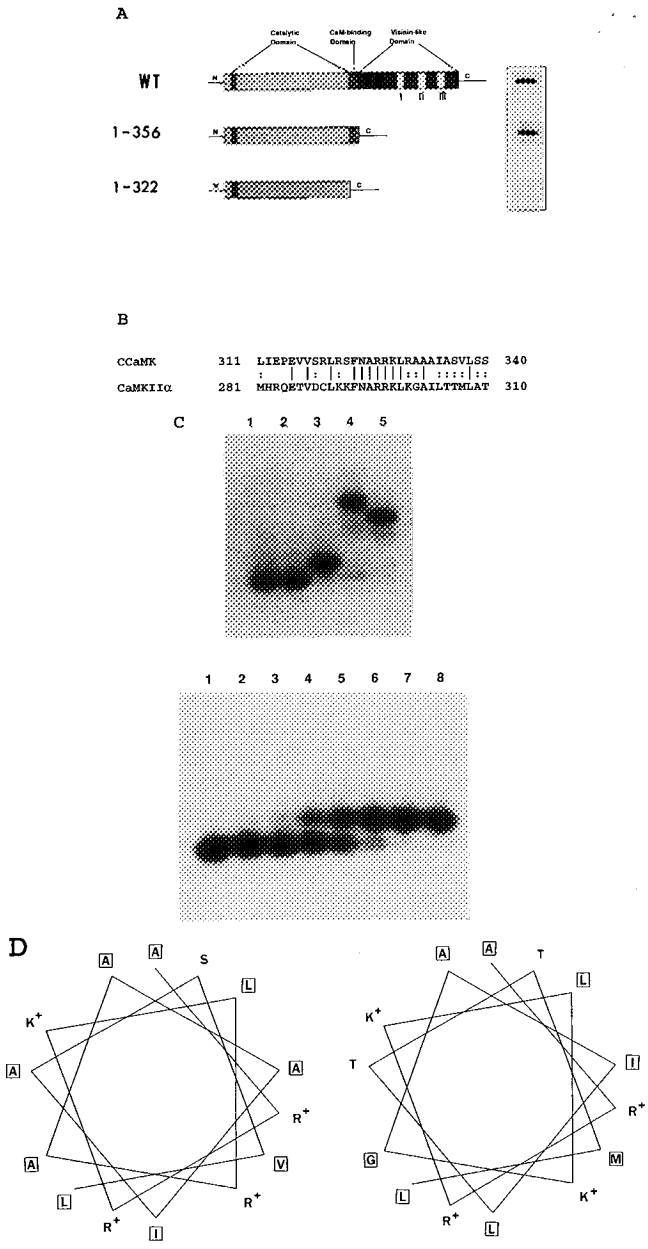

FIG. 3. **Identification of calmodulin binding site of CCaMK.** *A*, schematic diagram of wild-type and truncation mutants of CCaMK, which were used for ^{35}S-calmodulin binding assays are shown on the *left*. The mutants 1–356 and 1–322 represent CCaMK lacking the visinin-like domain and both visinin-like and calmodulin-binding domains. *E. coli*-expressed wild-type and mutant CCaMKs were electrophoresed on SDS-polyacrylamide gel and transferred onto nitrocellulose filter. The excised bands containing the expressed proteins were subjected to ^{35}S-calmodulin binding assay. The autoradiogram is shown on the *right* of each diagram (*boxed area*). The radioactivity (cpm) of bound ^{35}S-calmodulin was 11,600 for wild-type, 12,500 for the mutant 1–356, and 99 for the mutant 1–322, respectively. *B*, comparison of amino acid sequences surrounding the putative calmodulin-binding sites of CCaMK and α subunit of CaMK II. *C*, calmodulin binding to the synthetic peptides in a gel mobility-shift assay. Nondenaturing gel electrophoresis was performed in the presence of 0.5 mM $CaCl_2$. *Top, lane 1*, calmodulin alone (100 pmol); *lanes 2–5*, a mixture of calmodulin (100 pmol) and each of the following peptides (1 nmol): *lane 2*, CCaMK 328–340; *lane 3*, CCaMK 322–340; *lane 4*, 317–340; and *lane 5*, 311–340. *Bottom*, mixtures of calmodulin and CCaMK peptide 322–340 at different molar ratios. *Lane 1*, calmodulin alone (100 pmol); *lanes 2–8*, calmodulin (100 pmol) and 40, 80, 160, 240, 320, 480, and 640 pmol of the peptide 322–340. The bands of calmodulin and calmodulin-peptide complex were visualized by staining with Coomassie Brilliant Blue. *D*, helical wheel projection of calmodulin-binding sequences in CCaMK (*left*) and CaMKIIα (*right*). Hydrophobic amino acid residues are *boxed*. Basic amino acid residues are marked with (+).

like domain protein induces a conformational change. To verify that the EF-hand motifs in the visinin-like domain are responsible for the Ca^{2+}-dependent mobility shift, site-directed mutants of the visinin-like domain protein were created. Each of the EF-hands (I, II, and III) were mutated by replacing the amino acid residue at the $-x$ position (D417A, S453A, and T495A) in the EF-hands (Fig. 5*A*), which are known to be primary determinants of the Ca^{2+} dissociation rate (30). The mutant in which all three EF-hands are mutated was expressed in *E. coli* and purified, and the protein was also analyzed by SDS-PAGE in the presence of Ca^{2+}. The visinin-like protein mutated in the EF-hand I migrated at a similar position to the wild-type protein, suggesting that this site may not be functional. However, mutations in EF-hands II and III shifted the mobility of the protein toward the higher molecular weight. The mutant of the EF-hand III migrated to a similar position to the protein in which all three EF-hands are mutated (Fig. 5*B*). The migration of EF-hand III mutant in the presence of Ca^{2+} was also similar to the wild-type protein in the absence of Ca^{2+}. These results suggest that Ca^{2+} binding to the EF-hands II and III contribute to the Ca^{2+}-dependent mobility shift of the visinin-like domain protein. Removal of Ca^{2+} by EGTA shifts the mobility of all the mutant proteins to similar positions toward the higher molecular weight (data not shown).

To study the role of the visinin-like domain in Ca^{2+}-stimulated autophosphorylation, the CCaMK mutant 1–356 lacking the visinin-like domain was used for autophosphorylation and substrate phosphorylation. Autophosphorylation of mutant 1–356 was not stimulated by Ca^{2+} (Fig. 6, *A* and *C*); however, it retained Ca^{2+}/calmodulin-dependent kinase activity at a substantially reduced level (Fig. 6, *B* and *D*). This indicates that the visinin-like domain is required for Ca^{2+}-stimulated autophosphorylation as well as for maximal substrate phosphorylation.

In order to understand the significance of Ca^{2+}-stimulated autophosphorylation, the autophosphorylated CCaMK was used to study its effect on substrate phosphorylation. First we attempted to study the activity of the autophosphorylated CCaMK using histone IIAS as a substrate. However, in the presence of histone IIAS, calmodulin did not suppress the Ca^{2+}-dependent autophosphorylation of CCaMK. It is probable that histone IIAS was interacting with acidic proteins such as calmodulin and the visinin-like domain of CCaMK. Therefore, we used GS peptide as a substrate for studying the activity of autophosphorylated CCaMK. The rate of phosphorylation of the GS peptide by unphosphorylated CCaMK was stimulated by increasing concentrations of calmodulin, but the maximal stimulation was only 3–4-fold higher as compared with the basal activity. However, when autophosphorylated CCaMK was used, calmodulin stimulated the rate of phosphorylation of the GS peptide with similar kinetics as histone IIAS (Fig. 7*A*). To study the effect of autophosphorylation on kinase activity using GS peptide as substrate, we compared Ca^{2+}/calmodulin-dependent and Ca^{2+}/calmodulin-independent activity of autophosphorylated to unphosphorylated CCaMKs. Autophosphorylated CCaMK exhibits approximately 5-fold increased Ca^{2+}/calmodulin-dependent kinase activity as compared with the unphosphorylated enzyme. The maximal stimulation of autophosphorylated CCaMK by Ca^{2+}/calmodulin was 20–25-fold as compared with the EGTA control (Fig. 7*B*). Ca^{2+}/calmodulin-independent activity was not significantly affected by autophosphorylation. These results suggest that Ca^{2+}-induced autophosphorylation stimulates Ca^{2+}/calmodulin-dependent activity of CCaMK.

DISCUSSION

This report provides the biochemical evidence for a Ca^{2+}/calmodulin-dependent protein kinase in plants. Although sev-

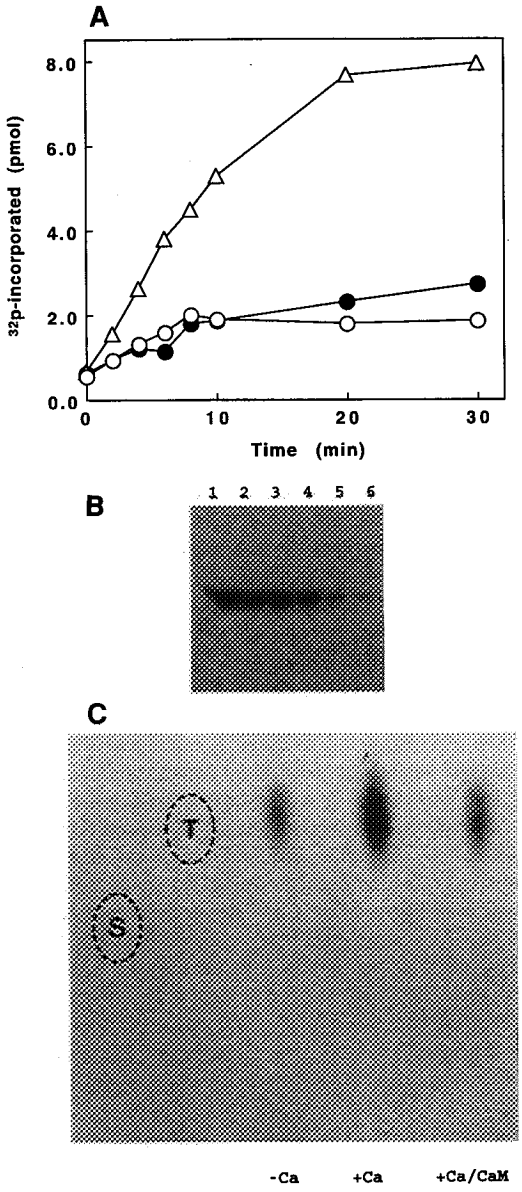

FIG. 4. **Effects of Ca^{2+} and Ca^{2+}/calmodulin on autophosphorylation of CCaMK.** *A*, time course of autophosphorylation of CCaMK in the presence of 2.5 mM EGTA (●) or 0.5 mM $CaCl_2$ (△) or 0.5 mM $CaCl_2$ and 1 μM calmodulin (○). The autophosphorylation is presented as pmol of ^{32}P incorporated per 21.4 pmol of CCaMK. *B*, effect of calmodulin on Ca^{2+}-dependent autophosphorylation of CCaMK. CCaMK was autophosphorylated in the presence of $CaCl_2$ (0.5 mM) and increasing concentrations of calmodulin. *Lane 1*, +$CaCl_2$, (0.5 mM); *lanes 2–6*, +$CaCl_2$ (0.5 mM) and 60, 120, 240, 360, and 480 nM calmodulin respectively. *C*, phosphoamino acid analysis of autophosphorylated CCaMK. CCaMK (200 ng) was autophosphorylated either in the presence of 2.5 mM EGTA (*−Ca*), 0.5 mM $CaCl_2$ (*+Ca*) or 0.5 mM $CaCl_2$ plus 1 μM calmodulin (*+Ca/CaM*). Autophosphorylated CCaMK was subjected to phosphoamino acid analysis. The positions of phosphoserine (*S*) and phosphothreonine (*T*) are marked.

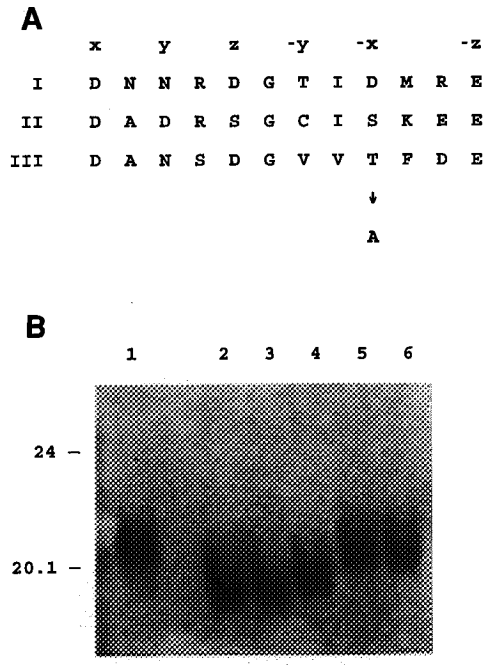

FIG. 5. **Binding of Ca^{2+} to visinin-like domain of CCaMK.** *A*, amino acid sequences of the three EF-hand motifs in the visinin-like domain of CCaMK. Six Ca^{2+} ligating residues denoted as *x*, *y*, *z*, *−y*, *−x*, *−z* are marked. Site-directed mutants were prepared by substituting the amino acid residues at *−x* position with alanine (*A*). *B*, Ca^{2+}-dependent mobility shift of wild-type and site-directed mutants of visinin-like domain protein. *E. coli*-expressed recombinant visinin-like domain proteins were electrophoresed on 14% SDS-polyacrylamide gel. In the presence of 2.5 mM EGTA (*lane 1*) or 0.5 mM $CaCl_2$ (*lanes 2–6*). Wild-type protein (*lanes 1* and *2*), proteins mutated in the EF-hand I (*lane 3*), EF-hand II (*lane 4*), EF-hand III (*lane 5*), and all three EF-hands (*lane 6*) are shown.

eral Ca^{2+}/calmodulin-dependent kinases have been characterized from animal systems (10), CCaMK is the only plant kinase whose activity is regulated by both Ca^{2+} and Ca^{2+}/calmodulin. Among the substrates tested, histone IIAS and synthetic GS peptide are the most efficient phosphate acceptors. CCaMK exhibits a higher K_a value (150–200 nM) for calmodulin (Figs. 1*A* and 7*A*) compared with CaMKII (20–100 nM) (31) and CaMKIV (26–150 nM) (32, 33), indicating that plant kinase requires a higher concentration of calmodulin for its activity. This is probably due to a higher dissociation constant of calmodulin for CCaMK (55 nM) than for animal Ca^{2+}/calmodulin-dependent protein kinases (1–10 nM) (34). ^{35}S-Labeled calmodulin binding and peptide binding assays revealed that calmodulin binding site of CCaMK is present between amino acid residues 322 and 340 (Fig. 3). This region has homology to animal CaMKII, with conserved basic (Arg-326, Arg-327, and Lys-328) as well as hydrophobic (Phe-323, Ala-325, Ala-332, and Leu-338) amino acid residues.

The visinin-like Ca^{2+}-binding domain, a novel feature of CCaMK, is not known to exist in other protein kinases. The visinin-like domain contains three EF-hand motifs (Fig. 5*A*) similar to animal visinin-like proteins. Frequenin, neurocalcin, and visinin-like proteins are known to be members of Ca^{2+}-sensitive guanylyl cyclase activators that are involved in cation channel regulation in neuronal tissues (35). Visinin-like proteins typically contain three conserved EF-hand motifs, each with different affinities to Ca^{2+} (36, 37). The Ca^{2+}-dependent mobility shift assay suggests that binding of Ca^{2+} to the EF-hands II and III is important for inducing conformational changes in the visinin-like domain of CCaMK (Fig. 5*B*). Ca^{2+}-induced conformational change in the visinin-like domain may be critical for regulation of CCaMK activity. The CCaMK mutant 1–356 lacking this domain did not show Ca^{2+}-dependent autophosphorylation. The mutant 1–356 also exhibited reduced activity as compared with the wild-type enzyme, suggesting that the visinin-like domain is required for the maximal activation of CCaMK. It is unlikely that this reduced activity is due to lowered affinity of mutant 1–356 to calmodulin, since the saturation curve of ^{35}S-calmodulin binding for mutant 1–356 indicated that it has a similar K_d (60 nM) for calmodulin (data not shown). However, it is possible that the visinin-like domain

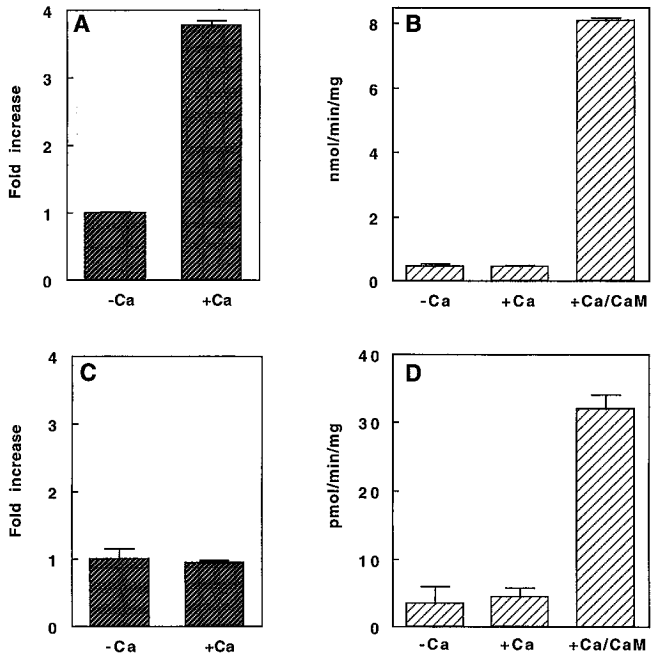

FIG. 6. **Comparison of enzyme activity of wild-type (*A* and *B*) and the truncated mutant (1–356) (*C* and *D*) of CCaMK.** Ca^{2+}-dependent autophosphorylation (*A* and *C*) and Ca^{2+}/calmodulin-dependent histone IIAS phosphorylation (*B* and *D*). The assays were carried out in the presence of 2.5 mM EGTA (*−Ca*), 0.5 mM $CaCl_2$ (*+Ca*), or 0.5 mM $CaCl_2$ and 1 μM calmodulin (*+Ca/CaM*).

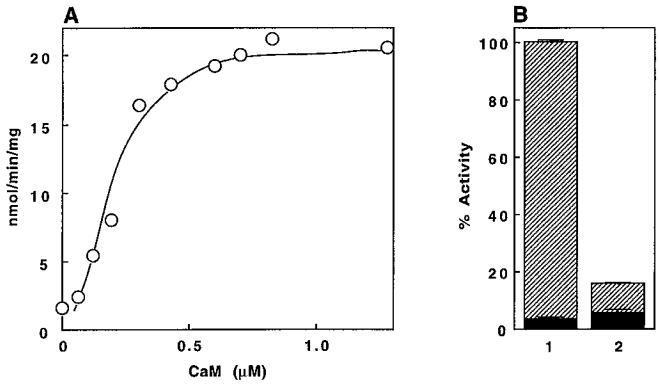

FIG. 7. **The effect of autophosphorylation of CCaMK on GS peptide phosphorylation.** *A*, effect of increasing concentrations of calmodulin on the GS peptide phosphorylation by autophosphorylated CCaMK. *B*, effect of CCaMK autophosphorylation on Ca^{2+}/calmodulin-dependent and -independent activity. Autophosphorylated and unphosphorylated enzymes were used for studying phosphorylation of the GS peptide. *Column 1*, CCaMK autophosphorylated in the presence of 0.5 mM $CaCl_2$ at 30 °C for 20 min and was used for Ca^{2+}/calmodulin-dependent GS peptide phosphorylation (*hatched bar*). *Column 2*, unphosphorylated enzyme incubated at 30 °C for 20 min and used for Ca^{2+}/calmodulin-dependent GS peptide phosphorylation (*hatched bar*). *Solid bars* represent the activity of autophosphorylated CCaMK (*column 1*) and unphosphorylated CCaMK (*column 2*) in the presence of 2.5 mM EGTA.

may stabilize the conformation of CCaMK, which is indispensable for its maximal activity.

The suppression of Ca^{2+}-dependent autophosphorylation of CCaMK by Ca^{2+}/calmodulin is intriguing. Phosphoamino acid analysis revealed that CCaMK autophosphorylation is due to the phosphorylation of the threonine residue(s) (Fig. 4*C*). Autophosphorylation of CCaMK increased its Ca^{2+}/calmodulin-dependent kinase activity by 5-fold (Fig. 7*B*). Ca^{2+}/calmodulin-dependent autophosphorylation of animal CaMKII at Thr-286 NH_2-terminal to the calmodulin binding site is known to stimulate Ca^{2+}-independent activity (11, 38, 39). In contrast, Ca^{2+}/calmodulin-independent basal autophosphorylation at Thr-305 and -306 within the calmodulin-binding site inactivates CaMKII by inhibiting its ability to bind calmodulin (40, 41). Although the calmodulin binding region of CCaMK has similarity to the calmodulin-binding region of CaMKII, there are no threonine residues around this area (Fig. 3*A*). The inhibition of the Ca^{2+}-stimulated CCaMK autophosphorylation by calmodulin may be due to the conformational change induced by the calmodulin binding to CCaMK (42). Inhibition of autophosphorylation by calmodulin is also reported in smooth muscle myosin light chain kinases (43), where all three phosphorylated residues are present in proximity to the calmodulin-binding site. The absence of threonine residues around the calmodulin-binding region of CCaMK suggests that the mechanism of CCaMK regulation by autophosphorylation is different from myosin light chain kinases and CaMKII.

Signal-induced changes in cytosolic Ca^{2+} concentration are believed to be important for many cellular processes in plants (2, 44, 45). Our results indicate that Ca^{2+} has a dual effect on the stimulation of CCaMK activity. In the presence of calmodulin, Ca^{2+} binds to calmodulin and stimulates CCaMK activity. In the absence of calmodulin, Ca^{2+} alone stimulates autophosphorylation of CCaMK, which further increases Ca^{2+}/calmodulin-dependent kinase activity (Fig. 7*B*).

Plants have multiple isoforms of calmodulin, and their expression is developmentally regulated and responsive to environmental signals (22, 46, 47). Plant calmodulin mRNA and protein are also reported to have a relatively rapid turnover rate in the cell (48). Signal-induced expression and rapid turnover suggest that there is a dynamic regulation of calmodulin *in vivo*. Therefore, it is likely that CCaMK activity is differentially controlled by signal-induced transient changes in free Ca^{2+} concentration and calmodulin. In plant cells, the Ca^{2+} concentration required for Ca^{2+}-dependent autophosphorylation and the Ca^{2+} concentration required for Ca^{2+}/calmodulin-dependent substrate phosphorylation may be different. In order to determine how the two regulatory domains control kinase activity, the site(s) of autophosphorylation and the critical concentrations of Ca^{2+} required for substrate phosphorylation and autophosphorylation need to be determined. These experiments are currently being carried out.

A unique feature of CCaMK is its stage-specific expression in developing anthers (20). We recently cloned a tobacco cDNA encoding a protein kinase with structural features similar to CCaMK, including calmodulin-binding and visinin-like Ca^{2+}-binding domains. Transgenic tobacco plants expressing the antisense RNA of this cDNA clone showed impaired pollen development,[2] indicating a crucial role for CCaMK in male gametophyte development.

The Ca^{2+} signaling pathway in plants is receiving considerable attention and is beginning to be unraveled at the molecular and biochemical levels (1, 2). The Ca^{2+}-signaling pathway mediated by Ca^{2+}/calmodulin-dependent kinases is well established in animals. Unfortunately, calmodulin-binding proteins, especially the kinases, have not been well characterized in plants. Therefore, the discovery of Ca^{2+}/calmodulin-dependent protein kinase and the elucidation of its biochemical properties will impact future studies on the role of calmodulin in Ca^{2+}-mediated signaling in plants.

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