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Functional Domains of Plant Chimeric Calcium/Calmodulin-Dependent Protein Kinase: Regulation by Autoinhibitory and Visinin-Like Domains[1]

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A novel calcium-binding calcium/calmodulin-dependent protein kinase (CCaMK) with a catalytic domain, calmodulin-binding domain, and a neural visinin-like domain was cloned and characterized from plants [Patil *et al.*, (1995) *Proc. Natl. Acad. Sci. USA* 92, 4797-4801; Takezawa *et al.* (1996) *J. Biol. Chem.* 271, 8126-8132]. The mechanisms of CCaMK activation by calcium and calcium/calmodulin were investigated using various deletion mutants. The use of deletion mutants of CCaMK lacking either one, two, or all three calcium-binding EF hands indicated that all three calcium-binding sites in the visinin-like domain were crucial for the full calcium/calmodulin-dependent kinase activity. As each calcium-binding EF hand was deleted, there was a gradual reduction in calcium/calmodulin-dependent kinase activity from 100 to 4%. Another mutant (amino acids 1-322) which lacks both the visinin-like domain containing three EF hands and the calmodulin-binding domain was constitutively active, indicating the presence of an autoinhibitory domain around the calmodulin-binding domain. By using various synthetic peptides and the constitutively active mutant, we have shown that CCaMK contains an autoinhibitory domain within the residues 322-340 which overlaps its calmodulin-binding domain. Kinetic studies with both ATP and the GS peptide substrate suggest that the autoinhibitory domain of CCaMK interacts only with the peptide substrate binding motif of the catalytic domain, but not with the ATP-binding motif.

Key words: autoinhibitory domain, calcium, calmodulin, visinin-like domain.

Calcium, a universal second messenger is known to regulate diverse cellular processes in eukaryotes. Calcium/calmodulin-regulated protein phosphorylation plays a crucial role in amplifying and diversifying the action of calcium-mediated signals. Although calcium/calmodulin-dependent protein phosphorylation is implicated in regulating a number of cellular processes in plants, not much is known about calcium/calmodulin-dependent kinases and their role in calcium-signaling. A chimeric plant calcium/calmodulin-dependent protein kinase (CCaMK) was recently cloned (*1*). This kinase is a novel serine/threonine kinase with two distinct regulatory domains within a single polypeptide: A calmodulin-binding domain and a calcium-binding visinin-like domain (*2*). Biochemical studies using *Escherichia coli*-expressed CCaMK show that the kinase activity is regulated by calcium and calcium/calmodulin. Calcium/calmodulin stimulates CCaMK activity by 20- to 25-fold, while calcium alone stimulates the autophosphorylation by 3- to 4-fold (*2*).

Studies on animal calcium/calmodulin-dependent protein kinases have revealed that the regulation of kinase activity by calcium/calmodulin is achieved by an intrasteric autoinhibitory domain (*3, 4*). These kinases are maintained in an inactive state in the absence of calcium/calmodulin by the interaction of the autoinhibitory domain with the catalytic site of the kinase domain. This autoinhibitory domain may contain a pseudosubstrate sequence that is recognized by the catalytic domain and competes with the exogenous substrate (*5*). The presence of an autoinhibitory domain has been demonstrated in calmodulin-dependent myosin light chain kinase (MLCK) (*6, 7*); calcium/calmodulin-dependent protein kinase I (CaMKI) (*8*); calcium/calmodulin-dependent protein kinase IV (CaMK IV) (*9*); and calcium/calmodulin-dependent protein kinase II (CaMKII) (*10*). In all these kinases, the autoinhibitory domain is mapped around the calmodulin-binding domain. Since there is relatively less amino acid sequence conservation around the calmodulin-binding and the autoinhibitory domain as compared to the catalytic domain, the autoinhibitory domain was identified only by structure/function studies.

The autoinhibitory domain of animal CaMKII has been identified by structure/function studies using a series of deletion/site directed mutants, synthetic autoinhibitory

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Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

peptides, or a partially proteolysed enzyme. The autoinhibitory domain (amino acids 281-302) partially overlaps the calmodulin-binding domain (amino acids 296-309) (*11*). This enzyme is kept in the inactive state in the absence of calcium/calmodulin because the autoinhibitory domain interacts with both the substrate peptide and the ATP binding motifs of the kinase. Once calcium/calmodulin binds to the enzyme these sites are relieved and the enzyme becomes active (*12, 13*). Partial cleavage of rat brain CaMKII with chymotrypsin is known to generate an active 30 kDa fragment, which exhibits calcium/calmodulin-independent activity (*14*). This 30 kDa fragment has been shown to contain an intact catalytic domain, but lacks the autoinhibitory domain and the calmodulin-binding domain (*14*). Synthetic peptides derived from the autoinhibitory domain efficiently blocked the activity of the 30 kDa catalytic fragment (*12*). Studies using deletion mutants expressed in *E. coli* also indicated that the removal of the autoinhibitory domain makes CaMKII constitutively active (*15*). Cruzalegui *et al.* (*16*) used a series of truncation and substitution mutants of CaMKII and identified amino acid residues critical for the autoinhibition. These studies suggested that basic amino acid residues (*12*) as well as the secondary structure (*17*), in the autoinhibitory domain are important determinants for maintaining the autoinhibitory state.

The calcium-binding visinin-like domain of CCaMK is unique and may add another calcium-sensing mechanism to the regulation of CCaMK. The role of the three calcium-binding EF hands within the visinin-like domain in the activation of CCaMK has been investigated. Various deletion mutants of CCaMK lacking one, two, or all three calcium-binding EF hands were used for this study. Since CCaMK activity is tightly regulated by calcium/calmodulin, the role of autoinhibition in the activation of CCaMK was investigated.

EXPERIMENTAL PROCEDURES

Construction of Deletion Mutants—The mutant 1-482 was created by replacing a 0.9 kb *Bam*HI fragment of wildtype expression plasmid pNY10 with a 380 bp *Bam*HI/*Bgl*II fragment corresponding to CCaMK amino acids 356 to 482. The deletion mutant 1-438 was generated by using an oligonucleotide 5′-GAAAACAGATCTGGAGAGC-3′. The mutant construct 1-356 was created by removing a 0.9 kb *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′-GCATTGAAAGATCTCA-GTCTAGAAAC-3′. The mutants 1-482, 1-438, 1-356 were cloned into the pET-3b expression vector and the mutant 1-322 was cloned into the pET-14b expression vector and expressed in *E. coli* strain BL21 (DE3) pLysS. These deletion mutants were expressed in *E. coli* and purified using either a calmodulin Sepharose affinity column (Pharmacia) or a Ni^{2+}-resin column (Novagen) and the protocols provided by the manufacturers.

Protein Kinase Assay—Phosphorylation assays (25 μl) were carried out at 30°C for 10 min 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), and 100 μM GS peptide (PLSRTLSVAAKK), in the presence of either 2.5 mM EGTA or indicated amounts of calcium or calcium/calmodulin. The reaction was terminated by spotting the mixture on p81 phosphocellulose filters (Whatman). The filters were washed in 75 mM phosphoric acid and ^{32}P incorporation was determined (*18*).

Gel Electrophoresis—SDS polyacrylamide gel electrophoresis was performed according to Laemmli (*19*). Non-denaturing gel electrophoresis was performed as described earlier (*2*).

Calmodulin Binding Assay to Synthetic Peptides—Synthetic peptides were prepared using the Applied Biosystem Peptide Synthesizer 431A in the laboratory of Bioanalysis and Biotechnology, Washington State University. The peptides were purified by reverse phase HPLC using a C8 column with a gradient of increasing CH_3CN concentration in 0.1% TFA as a solvent. Different amounts of purified synthetic peptides were incubated with 100 pmol (1.7 μg) of calmodulin in 20 μl of 20 mM Hepes (pH 7.5) for 5 min at room temperature and analyzed by non-denaturing polyacrylamide gel electrophoresis.

Biotinylated Calmodulin Overlay Assay—E. coli expressed recombinant fusion proteins were separated on the SDS polyacrylamide gel (*19*) and electrophoretically transferred onto a nitrocellulose filter (*20*). The filter was blocked in TBS containing 3% (w/v) nonfat dry milk at room temperature for 2 h. The filter was then incubated with 100 ng/ml biotinylated calmodulin (BRL) in binding buffer (TBS containing 1% w/v BSA, 1 mM $CaCl_2$) at room temperature for 2 h. After washing in TBS containing 1 mM $CaCl_2$, the filter was incubated with avidin conjugated alkaline phosphatase in the binding buffer for 2 h. The bound biotinylated calmodulin was detected by NTB/BCIP reagent (Amresco).

RESULTS

Reduced Activity of Deletion Mutants with a Truncated Visinin-Like Domain—In order to study the role of the neural visinin-like domain in the regulation of CCaMK activity, various deletion mutants were created. As shown in Fig. 1A, mutants were created lacking one, two, or all three calcium binding EF hands within the visinin-like domain. Another mutant (1-322) was also created which lacks both the visinin-like domain and the calmodulin-binding domain. These mutants were expressed in *E. coli* and the purified proteins were used to study the calcium-dependent mobility shift (Fig. 1B). The results indicate that the wildtype, as well as the mutants 1-482 and 1-438 show a calcium-dependent mobility shift. The mutants 1-356 and 1-322 did not show a calcium-dependent mobility shift. The calcium/calmodulin-dependent and independent activity of these mutants was studied. The results show that all of the mutants, except the mutant 1-322 were calcium/calmodulin-dependent for their kinase activity (Fig. 2). The mutant 1-482 lacking EF hand III showed a threefold reduction in calcium/calmodulin-dependent activity (37.7%) as compared to the wildtype. The activity of the mutant 1-438 lacking both EF hands II and III is even lower than the mutant 1-482 (28.7% of that of the wildtype). The mutant 1-356 lacking all three calcium-binding EF hands showed only 4% activity compared to the wildtype. Calcium/calmodulin-independent activity of these deletion mutants are 5% (wildtype), 4% (mutant 1-482),

and 0.3% (mutant 1-438), of that of the calcium/calmodulin-dependent activity of the wildtype kinase. These results show that disrupting portions of the visinin-like domain by deleting EF hands III, II, and I gradually reduced the total activity of CCaMK. However, the calcium/calmodulin-independent activity of these mutants varied slightly, indicating that the calcium-binding EF hands are crucial for the total calcium/calmodulin-dependent activity of CCaMK. Another mutant 1-322 also showed reduced activity

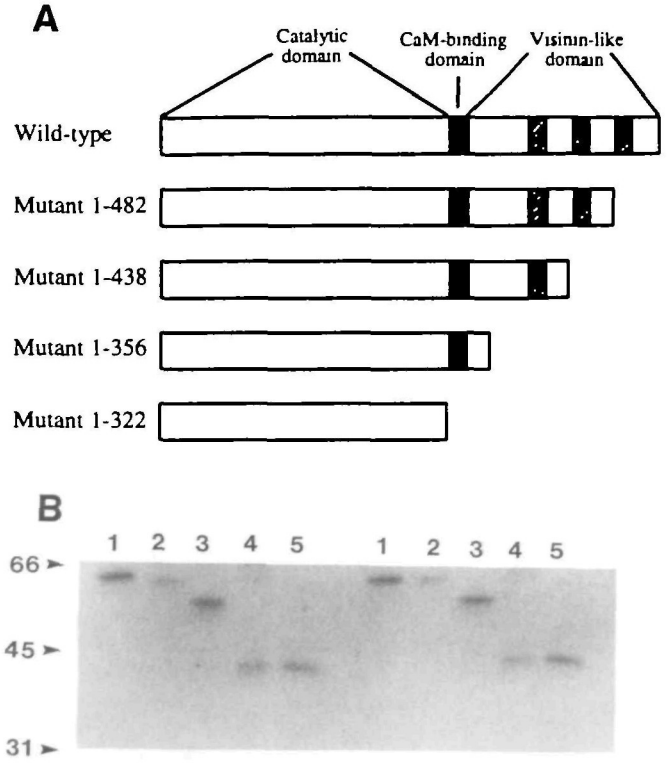

Fig. 1. **A: Schematic diagrams of wildtype CCaMK and the deletion mutants. B: SDS-PAGE of deletion mutants.** The purified proteins were analyzed by SDS-PAGE in the presence of calcium (1 mM) (left) or in the absence of calcium (2.5 mM EGTA) (right) and the protein bands were visualized by staining with Coomassie Brilliant Blue. Lane 1, wildtype; lane 2, mutant 1-482; lane 3, mutant 1-438; lane 4, mutant 1-356; and lane 5, mutant 1-322.

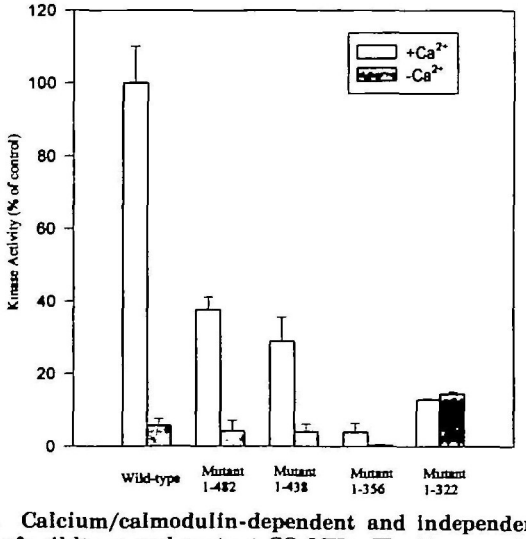

Fig. 2. **Calcium/calmodulin-dependent and independent activity of wildtype and mutant CCaMKs.** The kinase activity of CCaMK and its mutants were assayed in the presence of 0.5 mM $CaCl_2$ plus 1 μM calmodulin (open bars), or 2.5 mM EGTA (solid bars). The mean values and standard deviations were calculated from three independent experiments.

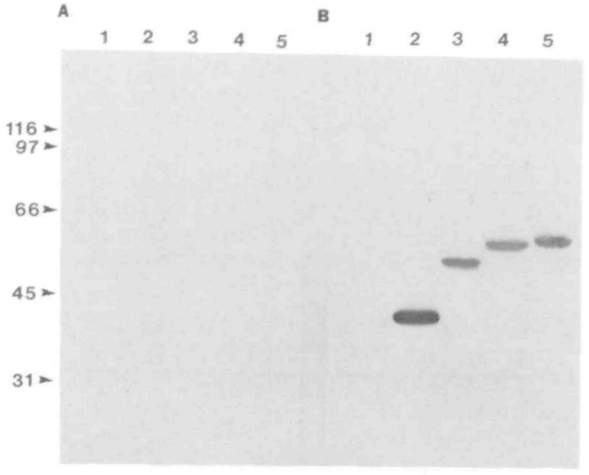

Fig. 3. **Calmodulin-binding assays of CCaMK and the mutants.** The lysates of IPTG-induced *E. coli* cell culture were separated on SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. Biotinylated calmodulin-binding assays were performed in the absence of calcium (2.5 mM EGTA) (A) and in the presence of calcium (1 mM $CaCl_2$) (B). Lanes 1 to 5 indicate that the crude lysates of mutant 1-322, 1-356, 1-438, 1-482, and wildtype, respectively.

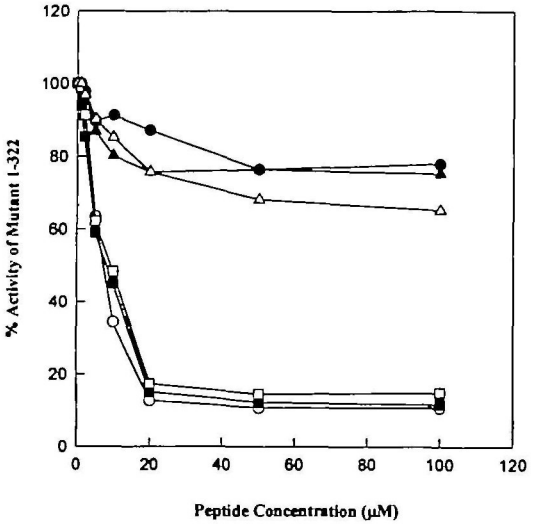

Fig. 4. **Effect of various synthetic autoinhibitory peptides on the activity of the constitutively active CCaMK mutant (1-322).** Mutant CCaMK lacking both the visinin-like domain and the calmodulin-binding domain (200 ng) was assayed in the presence of 0.5 mM calcium, 100 μM GS peptide, and indicated concentrations of synthetic peptides corresponding to amino acids 328 to 340 (●), 322-340 (○), 317-340 (■), 311-340 (□), 322-333 (▲) or 317-333 (△) for 2 min at 30°C under standard assay condition. The activity is represented as a percentage of control activity without the inhibitory peptides.

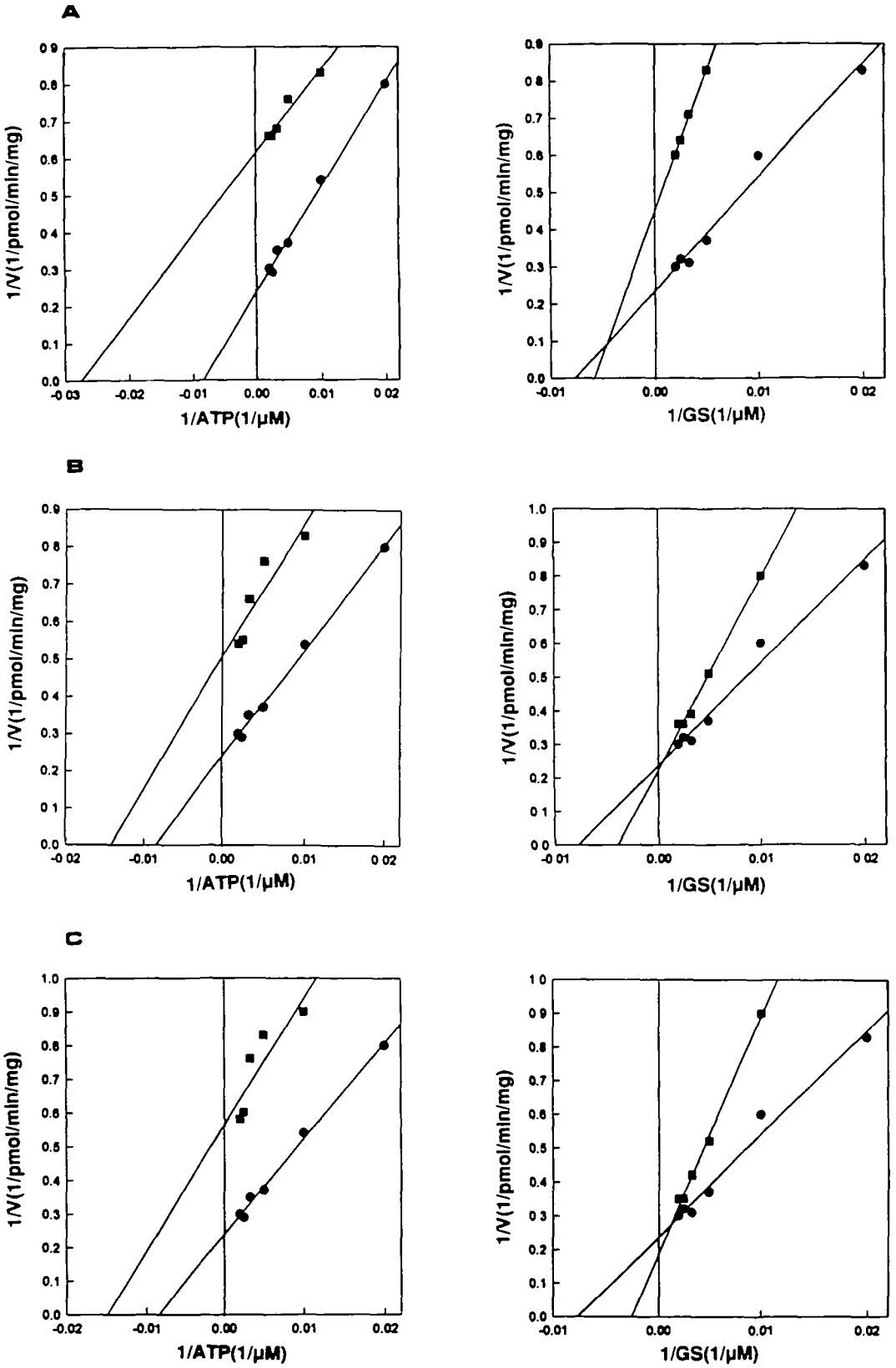

Fig. 5. **Kinetic analysis of CCaMK autoinhibition.** Mutant CCaMK (1-322) was assayed either in the presence (■) or absence (●) of 10 μM of peptide 322-340 (A), 317-340 (B), or 311-340 (C) under the standard assay conditions except for the concentration of substrates. For titration of the GS peptide (right panel), 500 μM of [γ^{32}P]-ATP and 50-500 μM of GS peptide were used. For titration of ATP (left panel) 500 μM of GS peptide and 50-500 μM of [γ^{32}P]ATP were used. The results were presented as double-reciprocal plots (Line Weaver-Burk).

TABLE I. **Kinetics of the interaction of autoinhibitory domain peptides with the mutant CCaMK (1-322).** IC_{50} values were determined by performing the kinase asssay of the constitutive mutant CCaMK (1-322) under standard conditions with varying amounts of inhibitory peptides. For kinetic studies, mutant CCaMK (1-322) was assayed in the presence or absence of inhibitory peptides. ATP and GS peptide substrates titrations were performed by keeping the concentration of one constant (500 μM) and varying the other (50 to 500 μM).

Peptides		IC_{50} (μM)	Type of inhibition	
			GS peptide	ATP
(311-340)	LIEPEVVSRLRSFNARRKLRAAAIASVLSS	9	Competitive	Uncompetitive
(317-340)	VSRLRSFNARRKLRAAAIASVLSS	8	Competitive	Uncompetitive
(322-340)	SFNARRKLRAAAIASVLSS	10	Mixed	Uncompetitive
(328-340)	KLRAAAIASVLSS	–	–	–
(322-333)	SFNARRKLRAAA	–	–	–
(317-333)	VSRLRSFNARRKLRAAA	–	–	–

(13 to 15% as compared to the wildtype). However, this activity is calcium/calmodulin-independent (Fig. 2). Calmodulin-binding of mutants 1-482 and 1-438 was studied to test whether the reduced calcium/calmodulin-dependent activity is due to altered calmodulin binding. All the mutants, except the mutant 1-322 that lacks the calmodulin-binding domain bind calmodulin in a calcium-dependent manner (Fig. 3). Crude protein extract from the induced *E. coli* was used for these experiments. These results indicate that the reduced activity of the mutants is not due to their inability to bind calmodulin.

Autoinhibitory Domain of CCaMK—The calcium-independent activity of mutant 1-322 is most likely due to the disruption of an autoinhibitory domain that was present in the wildtype and mutants 1-482 and 1-438 (Fig. 2). In addition, the fact that the mutant 1-322 did not bind calmodulin (Fig. 3), indicates the presence of an autoinhibitory sequence around the calmodulin-binding region which has been deleted or impaired in the mutant 1-322.

To verify the presence of an autoinhibitory domain around the calmodulin binding domain, a synthetic calmodulin-binding peptide 311-340 (*2*) was used in the kinase assay of the mutant 1-322. As shown in Fig. 4, this peptide inhibits the activity of mutant 1-322 in a concentration-dependent manner with IC_{50} of 9 μM. In order to dissect the autoinhibitory domain, various lengths of synthetic peptides were made and used in the assay of mutant CCaMK (1-322). Different concentrations of the inhibitor peptides were added to the reaction mixture and assayed under standard assay conditions. The results indicate that the peptides 311-340, 317-340, and 322-340 are effective in inhibiting the constitutive kinase activity with IC_{50} values of 9, 8, and 10 μM, respectively; whereas peptides 328-340, 317-333, and 322-333 showed only a slight effect (Fig. 4). Since the peptides 311-340, 317-340, and 322-340 bind calmodulin, the effects of these peptides on the wildtype CCaMK activity were not studied. The effects of these peptides on the wildtype CCaMK activity may be the result of sequestration of calmodulin by the peptides, rather than a direct effect of the peptides on the enzyme. These results indicate that amino acids 322-340 are the minimum length required for the effective inhibition of the activity of the mutant CCaMK (1-322). In order to understand the mechanism of autoinhibition in CCaMK, kinetic studies were performed with ATP and GS peptide substrate. Mutant CCaMK (1-322) was assayed with or without inhibitor peptides and increasing concentrations of ATP or GS peptide. The inhibition of mutant 1-322 by the peptides 311-340, 317-340, 322-340 were uncompetitive with ATP, but competitive with GS peptide (Fig. 5). A

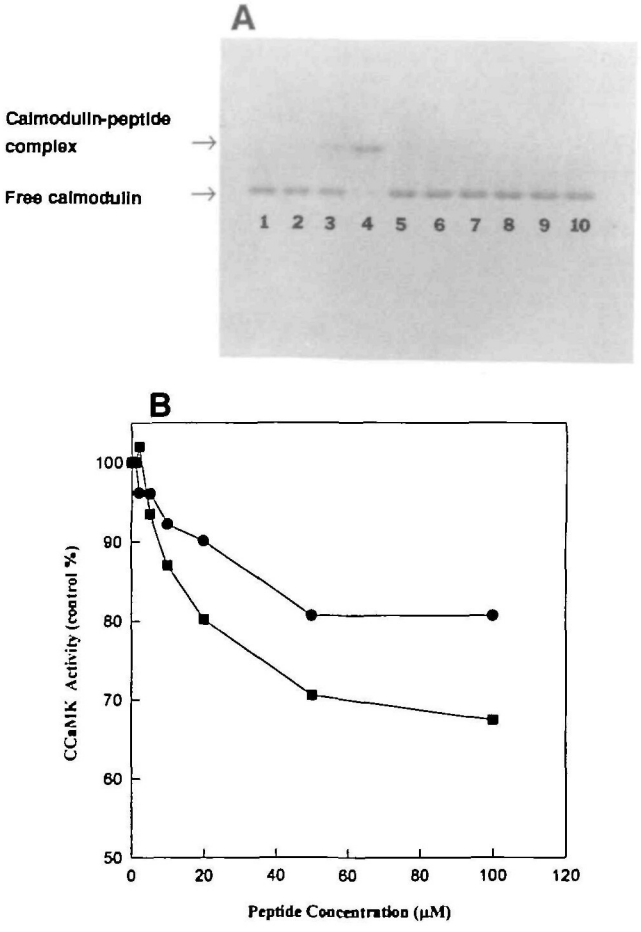

Fig. 6. **A: Calmodulin-binding to synthetic autoinhibitory peptides.** Spinach calmodulin (100 pmol) was mixed with indicated amounts of synthetic peptides in 20 mM HEPES in 20 μl and incubated for 5 min at room temperature and separated on a nondenaturing gel. The peptide calmodulin complex and free calmodulin were visualized by staining with Coomassie Brilliant Blue. Lane 1, calmodulin without peptides; lanes 2-4, 100 pmol of calmodulin with 20, 100, 250 pmol of peptide 317-340; lanes 5-7, 100 pmol of calmodulin with 40, 200, 400 pmol of peptide 322-333; lanes 8-10, 100 pmol of calmodulin with 40, 200, 400 pmol of peptide 317-333. **B: The effect of synthetic peptides on the activity of wildtype CCaMK.** One hundred nanograms of wildtype CCaMK was assayed under the standard assay conditions with 0.5 mM calcium and 1 μM calmodulin, and indicated concentrations of peptide 322-333 (●) or 317-333 (■). Activity is represented as a percentage of the control activity (without the inhibitory peptides).

summary of the kinetic studies using both ATP and GS peptide are shown in Table I. These results suggest that CCaMK has an autoinhibitory domain that has a different mechanism of action as compared to other calmodulin-dependent kinases.

A comparison of sequences of the autoinhibitory domain of CaMKII, CaMKIV, and CCaMK are shown in Fig. 7. It is clear that although these sequences have high homology within the calmodulin-binding domain, the N-terminal half of the autoinhibitory domains of these kinases is highly variable. More importantly, CaMKII and CaMKIV contain H^{282} which is absent in CCaMK.

In order to study the effect of synthetic autoinhibitory domain peptides on wildtype CCaMK activity, these peptides were checked for their calmodulin-binding efficiency. It was previously shown that peptides 311-340, 317-340, and 322-340, but not 328-340 bind calmodulin in a gel mobility shift assay (*2*). A similar calmodulin binding assay indicated that peptides 322-333 and 317-333 did not bind calmodulin (Fig. 6A). When these peptides were used in the assay of wildtype CCaMK, they did not significantly inhibit the calcium/calmodulin-dependent kinase activity. The peptide 322-333 inhibited wildtype CCaMK activity only by 20% at 100 μM; whereas, 100 μM of peptide 317-333 inhibited the activity by 32.5% (Fig. 6B). Even at higher concentrations above 100 μM, these peptides were ineffective in inhibiting the activity of CCaMK any further. These results indicate that the autoinhibitory domain of CCaMK overlaps its calmodulin-binding domain since only the peptides which bind calmodulin were effective in inhibiting the activity of the constitutive mutant CCaMK.

DISCUSSION

The deletion of each of the three calcium-binding EF hand motifs from CCaMK inhibited its calcium/calmodulin-dependent activity (Fig. 2). When the entire visinin-like domain was deleted, CCaMK activity was shown to drop to 4% of the wildtype activity, suggesting that the calcium-binding visinin-like domain of CCaMK plays a crucial role in the regulation of its calcium/calmodulin-dependent activity. However, binding of calmodulin to the mutants was not significantly affected by removal of the calcium-binding EF hands in the visinin-like domain. Since crude extracts were used for this assay and the amount of proteins used were not quantitated, the results did not indicate the affinity of each mutant to calmodulin. However, the results show that all the mutants bind calmodulin, thus indicating that the removal of the visinin domain does not lead to the loss of the mutants' ability to bind calmodulin.

The visinin-like domain of CCaMK can be contrasted with the calmodulin-like domain of calcium-dependent, calmodulin-independent protein kinase (CDPK) in plants. CDPK is also called a calmodulin-like domain kinase, since it has a COOH terminal regulatory domain that resembles calmodulin (*21*). This calmodulin-like domain containing four calcium-binding EF hand motifs acts like calmodulin and stimulates kinase activity upon binding calcium. Removal of the calmodulin-like domain from CDPK generates an inactive enzyme (*22*). Interestingly, the sequence comparison revealed that the calmodulin-like domain of CDPK and the visinin-like domain of CCaMK shared only 25% amino acid identity (*1*). Nevertheless, functional roles of these two regulatory domains may be quite different because CCaMK requires both calcium and calmodulin for its activation, while CDPK requires calcium alone. Furthermore, the *E. coli* expressed visinin-like domain protein of CCaMK does not bind the calmodulin-binding peptide (311-340 of CCaMK; data not shown), suggesting that the visinin-like domain does not act like the calmodulin-like domain of CDPK.

The visinin-like domain of CCaMK has a distinct role in autophosphorylation that substantiates maximal enzyme activity, which is also different from the COOH terminal domains of CaMKI and CaMKII in animals. Removal of the COOH terminal association domain of recombinant CaMKII has little effect on its calcium/calmodulin-dependent activity (*15*). Recombinant CaMKI lacking its COOH terminal portion also has similar calcium/calmodulin-dependent activity as compared to the wildtype enzyme (*8*). The functional role of the visinin-like domain might resemble the role of the regulatory subunit of calmodulin-dependent protein phosphatase (calcineurin) (*23*). Calcineurin is composed of a catalytic A subunit of protein phosphatase and a regulatory B subunit that binds calcium. The A subunit alone has very little phosphatase activity. Binding of calcium to the B subunit slightly stimulates phosphatase activity, but binding of calcium/calmodulin to the A subunit dramatically stimulates the activity to the maximal level. It is probable that the visinin-like domain acts as another calcium-binding regulatory subunit which is indispensable for maximal CCaMK activity. A putative calcium/calmodulin-dependent protein kinase gene has previously been isolated from apple (*24*), which has high homology to lily CCaMK except that it does not have a visinin-like domain. The fusion protein of apple kinase binds calmodulin in a calcium-dependent manner, but its phosphorylation activity and functional modulation by calcium/calmodulin have not been reported. This kinase itself may have very little activity just like the CCaMK mutants lacking the visinin-like domain and may need another regulatory subunit to have maximal calcium/calmodulin-dependent kinase activity.

Constitutive activity of the mutant 1-322 indicated the presence of an autoinhibitory domain around the calmodulin-binding domain (Figs. 1A, and 2). Various lengths of synthetic peptides were made based on the putative autoinhibitory domain sequence and are used in the assay of a deletion mutant of CCaMK (amino acids 1-322) which is constitutively active. The autoinhibitory domain of CCaMK is different from other known calmodulin-dependent kinases. Studies using synthetic peptides revealed that the peptide 322-340 is the minimum length required for the inhibition of the constitutive mutant of CCaMK 1-322 (Fig. 4). Previous characterization revealed (*2*) that the calmodulin-binding domain of CCaMK lies between the amino acid resides (322-340). Current studies show that the autoinhibitory domain of CCaMK overlaps its calmodulin-binding domain. The autoinhibitory domains of CaMKII and CaMKIV overlap in the calmodulin-binding domain and extend further into the amino terminal of the calmodulin-binding domain as compared to the autoinhibitory domain of CCaMK. The peptide 322-340 was adequate for inhibition of the constitutive CCaMK mutant. The longer peptides extending both the N-terminus and the C-terminus of the calmodulin-binding domain did not improve

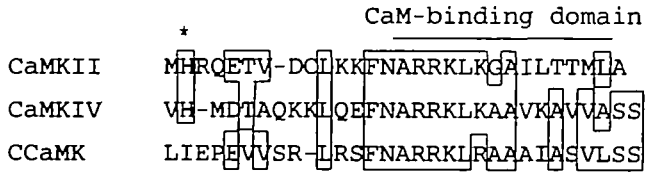

Fig. 7. **Putative autoinhibitory domain of CCaMK.** Alignment of the autoinhibitory domain sequence of CaMKII, CaMKIV, and the corresponding sequence in CCaMK. Identical residues are boxed. H^{282} is indicated by an asterisk.

the IC_{50} of the inhibition.

It is interesting that all three autoinhibitory peptides studied (311-340, 317-340, 322-340) were not competitive with ATP, but were competitive with the GS peptide substrate. The autoinhibitory domain of CaMKII is shown to be bifunctional since it interacts with both the ATP and peptide substrate binding motifs of the catalytic domain (*12*). In this respect, the mechanisms of the autoinhibitory domain of CCaMK may be similar to CaMKIV which was shown to be uncompetitive with ATP, but competitive with the substrate peptide (*9*). A comparison of sequences of the autoinhibitory domain of CaMKII, CaMKIV, and CCaMK are shown in Fig. 7. Although these sequences have high homology at the calmodulin-binding domain, the N-terminal halves of the autoinhibitory domains of these kinases were highly variable. It was shown that the H^{282} in the autoinhibitory domain of CaMKII was crucial for its interaction with the ATP binding region of the catalytic domain. The lack of a histidine residue in the corresponding region in CCaMK (Fig. 7) may explain the uncompetitiveness with ATP and consequently cause a relatively high (5%) calcium-independent activity of CCaMK. However, in the case of CaMKIV, although it has a histidine in a similar position, the autoinhibitory domain does not interact with the ATP binding region (*9*). These results suggest that apart from the H^{282}, conformation of the stretch of amino acids in this region may be crucial for interacting with the ATP binding domain. The significance of the CCaMK autoinhibitory domain that lacks an interaction with the ATP binding region of the catalytic domain is not clear.

Transgenic plants expressing the antisense RNA of CCaMK produced male sterile plants, indicating a major role for CCaMK in microsporogenesis (Liu and Poovaiah, unpublished data). The calcium signaling pathway in plants is beginning to be unraveled at the biochemical and molecular levels. Determining how CCaMK with its two distinct regulatory domains is involved in amplifying the calcium signal will provide valuable information in understanding the mechanisms involved in calcium-mediated signaling.

REFERENCES

1. Patil, S., Takezawa, D., and Poovaiah, B.W. (1995) Chimeric plant calcium/calmodulin-dependent protein kinase gene with a neural visinin-like calcium-binding domain. *Proc. Natl. Acad. Sci. USA* **92**, 4897-4901
2. Takezawa, D., Ramachandiran, S., Paranjape, V., and Poovaiah, B.W. (1996) Dual regulation of a chimeric plant serine threonine kinase by calcium and calmodulin. *J. Biol. Chem.* **271**, 8126-8132
3. Soderling, T.R. (1990) Protein kinases: Regulation by autoinhibitory domains. *J. Biol. Chem.* **265**, 1823-1836
4. Kemp, B.E., Parker, M.W., Hu, S., Tiganis, T., and House, C. (1994). Substrate and pseudosubstrate interactions with protein kinases: determinants of specificity. *Trends Biochem. Sci.* **19**, 440-443
5. Kemp, B.E., Pearson, R.B., Guerriero, V., Jr., Bagchi, I.C., and Means, A.R. (1987) The calmodulin binding domain of chicken smooth muscle myosin light chain kinase contains a pseudosubstrate sequence. *J. Biol. Chem.* **262**, 2542-2548
6. Krueger, J.K., Padre, R.C., and Stull, J.T. (1995) Intrasteric regulation of myosin light chain kinase. *J. Biol. Chem.* **270**, 16848-16853
7. Tanaka, M., Ikebe, R., Matsuura, M., and Ikebe, M. (1995) Pseudosubstrate sequence may not be critical for autoinhibition of smooth muscle myosin light chain kinase. *EMBO J.* **14**, 2839-2846
8. Yokokura, H., Picciotto, M.R., Nairn, A.C., and Hidaka, H. (1995) The regulatory region of calcium/calmodulin-dependent protein kinase I contains closely associated autoinhibitory and calmodulin-binding domains. *J. Biol. Chem.* **270**, 23851-23859
9. Tokumitsu, H., Brickey, D.A., Glod, J., Hidaka, H., Sikela, J., and Soderling, T.R. (1994) Activation mechanisms for Ca^{2+}/calmodulin-dependent protein kinase IV. *J. Biol. Chem.* **269**, 28640-28647
10. Brickey, D.A., Bann, J.G., Fong, Y.-L., Perrino, L., Brennan, R.G., and Soderling, T.R. (1994) Mutational analysis of the autoinhibitory domain of calmodulin kinase II. *J. Biol. Chem.* **269**, 29047-29054
11. Hanson, P.I. and Schulman, H. (1992) Neuronal Ca^{2+}/calmodulin-dependent protein kinases. *Annu. Rev. Biochem.* **61**, 559-601
12. Smith, M.K., Colbran, R.J., Brickey, D.A., and Soderling, T.R. (1992) Functional determinants in the autoinhibitory domain of calcium/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **267**, 1761-1768
13. Colbran, R.J., Smith, M.K., Schworer, C.M., Fong, Y.-L., and Soderling, T.R. (1989) Regulatory domain of calcium/calmodulin-dependent protein kinase II. Mechanism of inhibition and regulation by phosphorylation. *J. Biol. Chem.* **264**, 4800-4804
14. Colbran, R.J., Fong, Y.-L., Schworer, C.M., and Soderling, T.R. (1988) Regulatory interaction of the calmodulin-binding, inhibitory, and autophosphorylation domains of Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **263**, 18145-18151
15. Hagiwara, T., Ohsako, S., and Yamauchi, T. (1991) Studies on the regulatory domain of Ca^{2+}/calmodulin-dependent protein kinase II by expression of mutated cDNA's in *Escherichia coli*. *J. Biol. Chem.* **266**, 16401-16408
16. Cruzalegui, F.H., Kapiloff, M.S., Morfin, J.-P., Kemp, B.E., Rosenfeld, M.G., and Means, A.R. (1992) Regulation of intrasteric inhibition of the multifunctional calcium/calmodulin-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **89**, 12127-12131
17. Mukherji, S., Brickey, D.A., and Soderling, T.R. (1994) Mutational analysis of secondary structure in the autoinhibitory and autophosphorylation domains of calmodulin kinase II. *J. Biol. Chem.* **269**, 20733-20738
18. Roskoski, R., Jr. (1983) Assays of protein kinase. *Methods Enzymol.* **99**, 3-6
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
20. Towbin, H., Staehelin, T., and Gordon, T. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
21. Roberts, D.M., Lukas, T.J., and Watterson, D.M. (1986) Structure, function, and mechanism of action of calmodulin. *CRC Crit. Rev. Plant Sci.* **4**, 311-339
22. Harper, J.F, Huang, J.-F., and Lloyd, S.J. (1994) Genetic identification of an autoinhibitor in CDPK, a protein kinase with a calmodulin-like domain. *Biochemistry* **33**, 7267-7277
23. Kincaid, R. (1993) Calmodulin-dependent protein phosphatases from microorganisms to man. *Adv. Second Messenger Phosphoprotein Res.* **27**, 1-23
24. Watillon, B., Kettmann, R., Boxus, P., and Burny, A. (1993) A calcium/calmodulin-binding serine/threonine protein kinase homologous to the mammalian type II calcium/calmodulin-dependent protein kinase is expressed in plant cells. *Plant Physiol.* **101**, 1381-1384