Calcium-stimulated autophosphorylation site of plant chimeric calcium/calmodulin dependent protein kinase

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SUMMARY

The existence of two molecular switches regulating plant chimeric Ca\(^{2+}\)/calmodulin-dependent protein kinase (CCaMK), namely the c-terminal visinin-like domain acting as Ca\(^{2+}\)-sensitive molecular switch and calmodulin binding domain acting as Ca\(^{2+}\)-stimulated autophosphorylation sensitive molecular switch, have been described (Sathyanarayanan, P. V., Cremo, C. R., and Poovaiah, B. W., *J. Biol. Chem.* 275:30417-30422, 2000). Here we report the identification of Ca\(^{2+}\)-stimulated autophosphorylation site of CCaMK by MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time Of Flight) mass spectrometry. T267 was confirmed as the Ca\(^{2+}\)-stimulated autophosphorylation site by post source decay (PSD) experiments and by site directed mutagenesis. The purified T267A mutant form of CCaMK did not show Ca\(^{2+}\)-stimulated autophosphorylation, autophosphorylation-dependent variable calmodulin affinity or Ca\(^{2+}\)/calmodulin stimulation of kinase activity. Sequence comparison of CCaMK from monocotyledonous plant (lily) and dicotyledonous plant (tobacco) suggest that the autophosphorylation site is conserved. This is the first identification of a phosphorylation site specifically responding to activation by second messenger system (Ca\(^{2+}\) messenger system) in plants. Homology modeling of the kinase and calmodulin-binding domain of CCaMK with the crystal structure of CaMK I suggests that the Ca\(^{2+}\)-stimulated autophosphorylation site is located on the surface of the kinase and far from the catalytic site. Analysis of Ca\(^{2+}\)-stimulated
autophosphorylation with increasing concentration of CCaMK indicates the possibility that the Ca$^{2+}$-stimulated phosphorylation of occurs by an intermolecular mechanism.

**INTRODUCTION**

Intracellular calcium signals commonly exert their effects through the regulation of protein phosphorylation (2,3,4,5). These signaling events are orchestrated by calcium binding proteins that decode information contained in the spatial and temporal patterns of the Ca$^{2+}$ signals. In addition to calmodulin, a major transducer of Ca$^{2+}$ signals, plants contain Ca$^{2+}$/calmodulin (CaM)-dependent protein kinases and a large family of Ca$^{2+}$-dependent but CaM-independent protein kinases (CDPKs) and CDPK related protein kinases (CRKs). Ca$^{2+}$-dependent protein phosphorylation and Ca$^{2+}$/CaM-dependent protein phosphorylation are believed to participate in numerous aspects of plant growth and development (2,6). Several Ca$^{2+}$/CaM-dependent protein kinases have been known to exist in animal systems and numerous reports have appeared in the literature about their characterization and functional significance. However, not much is known about the Ca$^{2+}$/CaM-dependent protein kinases in plants (7-10). The chimeric Ca$^{2+}$/CaM-dependent protein kinase (CCaMK) reported from plants has a c-terminal visinin-like domain (9, 10) unlike all the other Ca$^{2+}$/CaM dependent protein kinases reported so far. CCaMK is expressed in a tissue-specific manner and it is developmentally regulated (11).
Apart from the c-terminal visinin-like domain, CCaMK has a serine-threonine kinase domain and an autoinhibitory domain overlapping with the CaM-binding domain. The CaM-binding domain of CCaMK is highly similar to mammalian Ca\textsuperscript{2+}/CaM dependent protein kinase II (12). Though there are several reports on Ca\textsuperscript{2+} and Ca\textsuperscript{2+}/CaM dependent protein phosphorylation in plants and their role in plant growth and development, the precise molecular nature of Ca\textsuperscript{2+} and Ca\textsuperscript{2+}/CaM dependent protein phosphorylation is not well understood.

In the present study, we identify and characterize the Ca\textsuperscript{2+}-stimulated autophosphorylation site of CCaMK by MALDI-TOF mass spectrometry and PSD experiments. The threonine 267 was found to be phosphorylated upon Ca\textsuperscript{2+}-stimulation and the T267A mutant did not show Ca\textsuperscript{2+}-stimulated autophosphorylation. Furthermore, Ca\textsuperscript{2+}/CaM-stimulated kinase activity and autophosphorylation-dependent CaM affinity changes were absent in the mutant. Identification of T267 as the Ca\textsuperscript{2+}-stimulated autophosphorylation site of CCaMK is an important step in the understanding of the molecular nature of transduction of Ca\textsuperscript{2+} signals into phosphorylation signals in plants. We show that a synthetic peptide (amino acid residues 257-340) containing the autophosphorylation site and CaM binding site could kinetically mimic the high affinity (phosphorylated) and low affinity (unphosphorylated) binding of CCaMK to CaM.
EXPERIMENTAL PROCEDURES

Expression and purification of CCaMK: CCaMK cDNA from Lily (*Lilium longiflorum* Thunb cv. Nellie white) and deletion mutants were cloned into the pET3b expression vector (Novagen, Inc) and expressed in *E. coli*. These proteins were purified as described previously (12).

Preparation of tryptic fragments of autophosphorylated CCaMK: Autophosphorylation of CCaMK was performed in the presence of 0.2 mM Ca2+ as described below. The phospholabeled protein was precipitated using chloroform/ methanol (13). The protein pellets were dried under a vacuum. The pellets were dissolved in 25 mM NH4HCO3 and freshly prepared trypsin (0.025 µg/µl in 25 mM NH4HCO3) was added (14). The reaction mixture was placed in a water bath at 37 °C for 18 hours. The tryptic fragments were then dried in a Speed Vac.

Immobilized metal chelate affinity chromatography (IMAC): IMAC for the purification of phosphopeptides was performed as described (14). IMAC Column was prepared using Ni-NTA agarose (Quiagen) (packed bed volume was 0.5 ml) in 10 ml Polyprep column (BioRad) using gravity flow. The column was washed with 2 ml of water followed by 2 ml of 0.1 M EDTA to remove bound divalent metal ions. The column was then washed sequentially with 2 ml water, 2 ml 0.1 M acetic acid, and 2 ml of 0.1 M ferric chloride in 0.1 M acetic acid to load the column with Fe3+ ions. 1 M acetic acid (2 ml) was then
used to elute unbound Fe$^{3+}$ ions. The tryptic digest of autophosphorylated CCaMK was dissolved in 100 µl of 0.2 M acetic acid and loaded onto IMAC column. The column was then washed with four column volumes of 0.1 M acetic acid followed by sequential washes with 2 column volumes of water and 0.1% ammonium acetate (adjusted to pH 8.0 with ammonium hydroxide). Bound peptides were eluted with four column volumes of 0.1% ammonium acetate adjusted to pH 9.5 with ammonium hydroxide. The peptides eluted using 0.1% ammonium acetate, pH 8.0 was used as a control in MALDI-TOF analysis because the majority of phosphopeptides are eluted at pH 9.5. The eluted fraction was dried under Speed Vac. The fractions were dissolved in 50 µl of 5% acetonitrile in water, containing 1% TFA and used for MALDI analysis.

**MALDI-TOF mass spectrometry:** MALDI-TOF mass spectrometry was performed on a PE Biosystems Voyager system DE/RP mass spectrometer equipped with a reflectron and nitrogen laser operating at 337 nm. Before analyzing the IMAC derived phosphopeptides, a computer generated tryptic peptide list (allowing 3 partials) was prepared from CCaMK amino acid sequence using the GPMAW 4.11 program (Lighthouse Data, Odense, Denmark). The IMAC fractions were analyzed in the linear positive ion delayed extraction mode using α-cyano-4-hydroxycinnamic acid (4HCCA) as the matrix. The IMAC derived peptides were further concentrated using C$_{18}$ ZipTip micro-columns (Millipore, Bedford, MA) using the manufacturer’s protocol for peptide concentration and clean up. The concentrated peptides were eluted from the Zip Tip with the matrix
solution (50/50 ACN/H2O with 0.25% TFA saturated with 4HCCA) and spotted directly onto the MALDI target plate and allowed to air dry. MALDI spectra were obtained using laser fluence approximately 20% above threshold. The accelerating voltage in the ion source was 25 kV, the delay time used was 150 ns, the grid voltage was set to 93.5% of the accelerating voltage and the guide wire voltage was set to 0.15% of the accelerating voltage. Typically 256 laser shots were averaged for each spectrum. MALDI spectra were calibrated externally using a standard peptide mixture. The spectra obtained were compared to the computer generated list of peptides to identify phosphopeptides by looking for a 80 Da shift corresponding to the addition of HPO3 to the –OH group of a serine, threonine, or tyrosine residue. The post source decay (PSD) experiment was performed in the reflectron positive ion delayed extraction mode, with an accelerating voltage of 25 kV, delay time of 200 ns, grid voltage of 65%, mirror voltage ratio of 1.12 and guide wire voltage of 0.1%. The timed ion selector (TIS) was set at m/z 2164 to obtain the PSD spectrum.

**Expression and purification of 35S-labeled CaM:** Potato calmodulin PCM1 cDNA cloned into the pET3b expression vector was labeled using 35S-methionine (New England Nuclear) as described (12). 35S-labelled CaM was purified from *E. coli* using phenyl-sepharose CL4B (Pharmacia) chromatography (12).

**Site-directed mutagenesis:** The site-directed mutagenesis was performed using the
QuikChangeTM site directed mutagenesis kit from Stratagene. The oligonucleotide primers used for generating T267A mutant (Thr mutated at Ala at the amino acid residue 267 in the CCaMK expression construct) were 5’- GATTTCAGCTTTGAGGAGCACGCGTGGAAGACCATAACTTCATC-3’ and 5’-GATGAAGTTATGTTATGGTCTTTCCACGCGTGCCTCTCAAAGCTGAAATC-3’. The sequence of the T267A mutant was confirmed by using the automated DNA sequencing facility available at Washington State University.

**CaM-binding assays:** CCaMK and mutant were incubated under autophosphorylating conditions (in the presence of Ca$^{2+}$) and in the absence of Ca$^{2+}$ (1). CaM-binding to CCaMK and T267A was assessed by spotting the reaction mixture onto nitrocellulose filters (Millipore) followed by incubating the filters with $^{35}$S-CaM (0.5X 10$^6$ cpm/µg) in binding buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl and 1% (w/v) nonfat dry milk plus either 1 mM Ca$^{2+}$ or 5 mM EGTA as described (1). An increase in the Ca$^{2+}$-dependent binding of $^{35}$S-CaM (autophosphorylated vs. unphosphorylated kinase and mutant) were plotted using SIGMAPLOT.

**Peptide-binding to Calmodulin:** A 84 amino acid peptide [257-340: lagd fsfeehtwkt itssakdlis sllsvdpykr ptandllkhp wvigdsakqe liepevvsrl rsfnarrklr aaaaaasvlss] containing the Ca$^{2+}$-stimulated
autophosphorylation site (T267) and the calmodulin binding site (311-340) was synthesized using Applied Biosystems peptide synthesizer 431A in the Laboratory of Bioanalysis and Biotechnology at Washington State University. This peptide was used as a substrate for the wild-type kinase in the phosphorylation assays. The phosphorylated peptides were purified using IMAC and used in the filter binding assays as described above.

**Autophosphorylation assays:** The autophosphorylation assay of CCaMK and mutant protein were carried out for 10 minutes at 30°C in the presence of 50 mM HEPES, pH 7.5, containing 10 mM magnesium acetate, 1 mM DTT, 1 mM (γ-32P)ATP and either 2.5 mM EGTA or 0.2 mM CaCl2. The reaction mixtures were analyzed on a 15% SDS-PAGE and the 32[P]PO4 incorporation was measured by isolating the protein bands from the gels and counting them using a scintillation counter. The data was analyzed using the parameter SAP (stimulated autophosphorylation index) (1) and plotted using SIGMAPLOT software (Jandel Scientific).

For the concentration dependence on autophosphorylation studies, CCaMK at various concentrations (0.18 µM-1.8 µM) were used in the autophosphorylation reactions as described. The 32[P]PO4 incorporation in the presence of 0.2 mM CaCl2 was measured using a scintillation counter as mentioned above. The regression analysis of data was performed using SIGMAPLOT. The fit to the data and data were plotted using the same software.
**Protein kinase assays:** GS peptide was used as a substrate to study kinase activity of wild-type and mutant CCaMK. Phosphorylation assays (20 µl) were carried out for 30 minutes at 30°C in the presence of 50 mM HEPES, pH 7.5, containing 10 mM magnesium acetate, 1 mM DTT, 1 mM ($\gamma$-32P)ATP and either 2.5 mM EGTA, 0.5 µM calmodulin, 100 µM GS peptide or 0.2 mM CaCl2, 0.5 µM calmodulin and 100 µM GS peptide (12). Spotting the reaction mixture onto 2.1 cm diameter WHATMAN P81 phosphocellulose filter circles terminated the reactions. The P81 filter circles were washed four times in 500 ml cold 0.5% phosphoric acid (10 minutes per wash). The filters were then washed with 200 ml acetone at room temperature for 5 minutes. The filter circles were dried at room temperature and 32[P]PO4 incorporation was measured using a scintillation counter. The fold increase in the 32[P]PO4 incorporation (Ca2+/CaM vs EGTA/CaM) is plotted as an indicator of kinase activity using SIGMAPLOT software.

**Calmodulin activation assays:** Substrate phosphorylation by the T267A mutant was carried out in 20 µl reactions for 30 minutes at 30°C in the presence of 50 mM HEPES, pH 7.5, containing 10 mM magnesium acetate, 1 mM DTT, 1 mM ($\gamma$-32P)ATP and either 2.5 mM EGTA and 100 µM histone IIAS or 0.2 mM CaCl2 and 100 µM histone IIAS (12) with increasing concentrations of CaM. The reactions were terminated by adding SDS-PAGE sample buffer and analyzed on a 10% SDS-PAGE. Proteins were
visualized by staining with Coomassie Brilliant Blue (Biorad). Incorporation of $^{32}$[P]PO$_4$ into the substrate was determined by counting the excised protein bands in a scintillation counter.

**Sequence analysis and Homology modeling:** The coordinates of calcium/calmodulin dependent protein kinase 1 (CaMK 1) crystal structure (15), PDB file 1A06, was obtained from Professor John Kuriyan’s laboratory web site (Rockefeller university). Modeling of kinase and calmodulin binding domains of CCaMK (residues 1-340) were carried out using MODELLER (16). The sequence alignment of CCaMK kinase and calmodulin domain and CaM kinase 1 was produced by CLUSTALW at the world wide web site of the European Bioinformatics institute. The two sequences have 31% sequence identity and 67% similarity determined by GAP program (GCG package). Amino acid sequences mammalian CaM kinases (17,18,19) were compared with CCaMK from plants (9,10) using CLUSTALW and the conserved regions were shaded by BOXSHADE program available at European Molecular Biology Net work web site.
RESULTS

MALDI-TOF MS Identification of phosphopeptides in tryptic peptides from autophosphorylated CCaMK: Phosphopeptides from the tryptic digests of autophosphorylated CCaMK were purified using IMAC and subjected to MALDI-TOF mass spectrometry. We could not detect any strong signals in the MALDI spectrum and the IMAC derived peptides were further concentrated using a C18 ZipTip. The ZipTip concentrated peptides were analyzed in the linear positive ion mode and several peaks were obtained. Tentatively we identified three phosphopeptides (Table 1) by comparing the m/z values of the peaks from the MALDI spectrum with the computer generated list. We could identify three tryptic peptides starting at residue 253 out of 4 predicted peptides having three or fewer cleavage sites. The major signal in our MALDI spectrum (Fig. 1a and Fig. 1b) correspond to [M+H]+ of peptide Q253-K269 with a single threonine as the only possible phosphorylation site since previous work (12) has shown that only threonines are phosphorylated upon Ca2+ stimulation. The peak (m/z 2167.7) was absent in the control (peptides eluted by 0.1% ammonium hydroxide, pH 8.0) and we identified Q253-K269 as a phosphopeptide. The other two phosphopeptides (residues Q253-K276 and Q253-K289) appeared as small peaks in the spectrum.

Post Source Decay Analysis: Tryptic peptide Q253-K269 containing T267 residue phosphorylated represented the strongest signal in the MALDI spectrum of IMAC derived peptides. This peak was further analyzed in the reflectron positive ion mode with
timed ion selector set at m/z 2164 to obtain the PSD spectrum. The PSD spectrum (Fig. 1c) showed the appearance of a peak at m/z of 2064.7 at high laser fluence (difference of 99.3 Da) suggesting the metastable loss of H$_3$PO$_4$ (loss of 98 Da (20)). The peak expected at -80 Da (loss of HPO$_3$) was more weakly observed.

**Preparation and characterization of T267A site-directed mutant:** The site-directed mutant T267A (threonine mutated to alanine) was prepared from the CCaMK expression vector using the site-directed mutagenesis kit and the mutation was confirmed by DNA sequencing. The T267A mutant was expressed in *E. Coli* and purified as described (12). Wild-type and mutant CCaMK were autophosphorylated in the presence of calcium and in the absence of calcium as described. The $^{32}$PPO$_4$ incorporation was measured and the Ca$^{2+}$-stimulation of autophosphorylation was expressed as SAP (stimulated autophosphorylation) index (1). SAP index represents the ratio of $^{32}$PPO$_4$ incorporation in the presence of Ca$^{2+}$ to that in the absence of Ca$^{2+}$. Figure 2 and Table II shows autophosphorylation of wild type and T267A mutant.

The interaction between calmodulin and the wild type and mutant form of the kinase was studied using $^{35}$S-CaM filter binding experiments. The autophosphorylated wild-type kinase showed about 7-fold increase in the $^{35}$S-CaM binding whereas phosphorylation dependent increase in the $^{35}$S-CaM binding was absent in the T267A mutant (Fig. 3).
determine the importance of T267 phosphorylation in increased affinity for CaM, a peptide (amino acid residues 247-340) containing the autophosphorylation site and calmodulin-binding site was used in the $^{35}$S-CaM filter binding experiments. The phosphorylated peptide showed about 5-fold increase in $^{35}$S-CaM binding as compared to the unphosphorylated peptide (Fig. 3).

The Ca$^{2+}$/CaM-stimulation of kinase activity of wild-type and mutant was tested using GS peptide as a substrate. The wild-type showed about 6 fold increase in the kinase activity in the presence of Ca$^{2+}$/CaM where as T267A mutant did not show any increase in kinase activity upon Ca$^{2+}$/CaM-stimulation (Fig. 4). CaM-dependent activation of the T267A kinase activity was studied by determining the $^{32}$P$	ext{PO}_4$ incorporation into histone IIAS by T267A in the presence of increasing concentrations of CaM (Fig. 5). Higher concentrations of CaM did not stimulate the kinase activity of T267A.

**Sequence comparison of CCaMK with other Ca$^{2+}$/CaM-dependent protein kinases:** Amino acid sequences corresponding to the kinase and CaM-binding domain of the three mammalian CaM kinases (CaM Kinase I (17), CaM kinase II (18) and CaM kinase IV (19)) were compared with plant chimeric kinase (NTCCaMK 1, NT CCaMK 2 from tobacco (9) and LICCaMK from lily (10)) using CLUSTALW program. The catalytic site sequences, activation loop and the CaM-binding sequences show many conserved regions (Fig. 6). The autophosphorylation site of the CCaMK from plants is marked with
Homology molecular modeling of the kinase and CaM-binding domain of CCaMK: The homology model of CCaMK is based on the 2.5 angstrom crystal structure of CaMK I (PDB file 1A06) and assumes that the two sequences share a common tertiary structure. This assumption is supported by the high degree of sequence identity. The resulting homology model of CCaMK reflects the structure of the template molecule, CaMK I, in positions of conserved sequence, which includes most of the protein. However, amino acids 1-9, 54-63, 164-181 and 317-374 are missing from the crystal structure and as a result the corresponding amino acids are less well defined in the homology model. Figure 7 shows the ribbon diagram of the resulting homology model with the region of interest highlighted by color. The red region in Figure 7 is the catalytic region, the yellow area is the peptide fragment determined to be phosphorylated by the MALDI/TOF experiments, and the threonine believed to be phosphorylated is shown in magenta.
DISCUSSION

The importance of Ca\textsuperscript{2+}-stimulated autophosphorylation in regulating the activity of chimeric Ca\textsuperscript{2+}/CaM-dependent protein kinase (CCaMK) was previously reported from our laboratory (1,12). An interesting consequence of Ca\textsuperscript{2+}-stimulated autophosphorylation of CCaMK is the significant increase in the affinity for calmodulin (1). Autophosphorylation dependent increase in affinity for calmodulin by CaMK II described as calmodulin trapping has been implicated in decoding of frequency dependent Ca\textsuperscript{2+} signals (3,4). The importance of CaMK II autophosphorylation in memory process has been described (21,22). However not much is known about specific residues phosphorylated or the significance of autophosphorylation of protein kinases in plants.

MALDI-TOF mass spectrometry is being widely used to study post-translational modifications in proteins (23-26). In this study, we have identified the Ca\textsuperscript{2+}-stimulated autophosphorylation site of chimeric CaM kinase (CCaMK) from plants using MALDI-TOF mass spectrometry. Table 1 shows the list of peptides identified (residues Q253-K269, Q253-K276 and Q253-K289). Fig 1a and Fig 1b show the linear positive ion MALDI spectrum of the IMAC derived peptides using 4HCCA as matrix. Only the strongest peak (m/z 2167.7) is shown. The other two phosphopeptides appeared as minor peaks in the MALDI spectrum. Previous reports (12) have indicated that only threonines are phosphorylated upon Ca\textsuperscript{2+}-stimulation of CCaMK. The 5-6 Da difference between
theoretical and observed m/z values was found consistently, despite repeated external recalibration (we had insufficient sample to attempt internal calibration). This is well outside the ±2 Da range that would be expected for our instrument in the mass range of our peptides. No common amino acid modification can account for this difference, and while a substitution of H266 to methionine could account for our observations, this mutation seems unlikely. Another possibility is a strong, uncorrected matrix-caused mass shift. We have seen a shift of this magnitude with labeled peptides containing a biotin moiety that have been separated with an avidin column (unpublished results), but never with peptides that have had a final C18 ZipTip clean up. The three peptides observed in the spectrum (Q253-K269, Q253-K276 and Q253-K289) contain 1, 3, 3 threonines respectively. Comparison of m/z values of the three peptides identified from the MALDI spectrum with the computer generated list of tryptic digests suggests that the three peptides observed are phospho-peptides. This corresponds to the phosphorylation of a single threonine (T267) as we did not observe mass difference for multiple phosphorylation (mass difference of multiples of 80 Da corresponds to multiple phosphorylation sites).

Post source decay experiment was conducted to confirm the presence of phosphorylated peptide. Fig. 1c shows PSD spectrum of peptide Q253-K269 (major peak observed in the MALDI spectrum) with the timed ion selector set at 2164 Da. The appearance of a peak at 2064 suggested loss of H3PO4 (loss of 98 Da) from the peptide Q253-K269. A peak corresponding to the loss of a phosphate (80 Da) was also observed in the spectrum. The
peaks corresponding to the loss of H$_3$PO$_4$ and HPO$_3$ confirmed that Q253-K269 is a phosphopeptide and that T267 is phosphorylated.

The phosphorylation site T267 was characterized by making a site-directed mutant T267A, threonine mutated to an unphosphorylatable amino acid, alanine. Ca$^{2+}$-stimulated autophosphorylation assays of T267A showed a SAP index value of 1, suggesting the absence of Ca$^{2+}$-stimulated autophosphorylation (Fig 2). This further supported the results from the MALDI experiments. We previously reported (1) that Ca$^{2+}$-stimulated autophosphorylation of CCaMK leads to a significant increase in the affinity for calmodulin. The mutant T267A was used to test this by $^{35}$S-CaM filter binding experiments. No significant increase in the $^{35}$S-CaM-binding was detected for T267A unlike the wild type, which showed about a 7-fold increase in $^{35}$S-CaM binding (Fig. 3).

Synthetic peptides have proved to be useful models for elucidating the mechanistic details of CaM binding to CaM kinase (44-50). Peptide (amino acid residues 257-340) containing the phosphorylation site (T267) and the CaM binding site (311-340) was synthesized to study the mechanism of activation of autophosphorylation by calcium. The $^{35}$S-CaM filter binding experiments showed a 5-fold increase in the $^{35}$S-CaM-binding to the phosphorylated form of the peptide in comparison to the unphosphorylated peptide.
(Fig. 3). This suggests that the peptide (residues 257-340) can kinetically mimic the low affinity (unphosphorylated) and high affinity (phosphorylated) binding of CCaMK to CaM. However the phosphorylated form of this peptide could not completely mimic the CaM-binding to the autophosphorylated kinase. This indicate that more amino acids are required in determining the phosphorylation dependent conformational changes in the CaM-binding region that may allow formation of additional interactions with CaM leading to a significant increase in affinity for CaM. The increased CaM affinity that can be seen by the peptide also suggest that the increased affinity due to autophosphorylation of CCaMK may not be due to an increased access of the CaM-binding domain. Furthermore, there may be direct interaction between the phosphate and CaM.

Mutant T267A was tested for Ca$^{2+}$/CaM -stimulation of kinase activity using GS peptide as substrate. It has been reported that Ca$^{2+}$/CaM -stimulated substrate phosphorylation by about 6-fold when GS peptide was used as a substrate (12). The P81 phosphocellulose experiment to study the kinase activity of T267A suggested the absence of Ca$^{2+}$/CaM -stimulated kinase activity (Fig. 4). Thus the T267A was found to be deficient in Ca$^{2+}$ -stimulated autophosphorylation, autophosphorylation dependent changes in the CaM affinity and Ca$^{2+}$/CaM -stimulated kinase activity, suggesting that the T267 as the amino acid phosphorylated upon the Ca$^{2+}$ -stimulation of the kinase.

If the increased affinity for CaM resulting from autophosphorylation is the only effect of
autophosphorylation, the T267A could be activated by higher concentrations of CaM. The increasing concentrations of CaM (0-4.8 µM) in the substrate phosphorylation reactions did not show a significant increase in the $^{32}\text{P}\text{PO}_4$ incorporation into histone IIAS by T267A (Fig. 5). There is some stimulation (~1.2 fold increase) of the kinase activity when the concentration of CaM was increased from 0-0.12 µM in the assay mixture but further increase in CaM concentrations did not show any increase in kinase activity. We have increased CaM concentrations up to 38.4 µM in the assay mixture (data not shown) but failed to observe an activation of T267A.

CaM can also bind to WT CCaMK in the absence of autophosphorylation with a Kd of ~55 nM (1, 12). However CaM does not stimulate the kinase activity in the absence of autophosphorylation (12). The fact that we did not observe activation of T267A mutant by increasing levels of CaM and that CaM binding to WT CCaMK does not activate the kinase activity in the absence of autophosphorylation suggest that there may be phosphorylation-dependent conformational changes that significantly alter the properties of CCaMK. Previous studies indicated that in the absence of autophosphorylation, CaM-binding affinities were not significantly altered in wild-type or CCaMK mutants in which different Ca$^{2+}$-binding sites (EF hands) were successively deleted (1). The conversion of an inactive form of kinase to maximally active kinase could be complex and that may not be achieved by simply increasing the concentration of CaM in the reaction. Thus, the Ca$^{2+}$-stimulated autophosphorylation has different effects on CCaMK including increased
CaM affinity and increased kinase activity.

T267 autophosphorylation site of CCaMK was further analyzed by comparing the amino acid sequences of CCaMK from plants with mammalian CaM dependent protein kinases such as CaM kinase I, CaM kinase II, and CaM kinase IV. Fig. 6 shows the alignment of the different CaM kinase sequences. Residues in the catalytic loop (RDLKPEN) and the activation loop (DFG….A/SPE) (27) found to be conserved in the CaM kinases shown in the figure. They also show high similarity in the CaM-binding region. Table III shows the autophosphorylation sites of different Ca\(^2+\)/CaM-dependent protein kinases. Though it appears from the Fig. 6 that the CaM kinases share significant homology in the catalytic site and CaM-binding regions, the molecular mechanism of calcium signal transduction could be different among different CaM-kinases as indicated by different phosphorylation sites. CaMK I is activated by phosphorylation in the activation loop (17,27,28). The Ca\(^2+\)/CaM dependent autophosphorylation site of CaMK II lies out side the activation loop but in the regulatory segment near to the CaM-binding region (15,29). Autophosphorylation of CaMK IV is also Ca\(^2+\)/CaM dependent and occurs on several serine residues (30,31). Both CaMK II and CaMK IV have the conserved autophosphorylation sequence, R-X-X-S/T. Sequence analysis suggests that the phosphorylation site of CCaMK (T267) is not in the conserved activation loop, but in the 10th subdomain of the serine-threonine protein kinase domain (32,33) of CCaMK (data not shown). Ca\(^2+\)-stimulated
autophosphorylation at T267 suggest a unique mechanism operating in the activation of CCaMK. This autophosphorylation site is found to be conserved across monocotyledonous and dicotyledonous plants (Fig. 6).

Homology modeling (34) of the kinase and calmodulin-binding domain of CCaMK to visualize the implications of phosphorylation site is shown in Figure 7. The observations suggest that the T267 phosphorylation site is located at the surface of the molecule, and far from the catalytic site. This suggests that T267 is more likely to be phosphorylated by intermolecular autophosphorylation upon Ca\(^{2+}\)-stimulation rather than an intramolecular autophosphorylation. However, it is possible that a large conformational change occurs upon Ca\(^{2+}\)-stimulation of the kinase such that T267 can enter the catalytic site leading to intramolecular autophosphorylation. The intermolecular autophosphorylation may also requires that the kinase exist as an oligomer as in the case of CaMK II (3,35). Considering the limitations of homology models, assigning a mechanism based solely on the molecular model is speculative. However, the homology model of CCaMK indicates that the autoinhibitory sequences can enter the kinase catalytic site and CaM-binding could lead to the removal of these autoinhibitory interactions and support previously published results about autoinhibition and activation of CCaMK (36). To test the possibility that Ca\(^{2+}\)-stimulated autophosphorylation occurs by inter-molecular or intra-molecular mechanism, concentration dependence of autophosphorylation of CCaMK was studied. If activation is determined by intra-molecular
autophosphorylation, the Ca\(^{2+}\)-stimulated autophosphorylation should vary in direct proportion to enzyme concentration. If activation is dependent on inter-molecular autophosphorylation, the Ca\(^{2+}\)-stimulated autophosphorylation will not vary in direct proportion to enzyme dilution, rather a cooperative relationship should be observed. Regression analysis of data in Fig. 8 indicates that for the different concentrations of C\(\text{CaMK}\) studied, change in autophosphorylation with respect to enzyme concentration was better fit to a curve than to a straight line. For 10 min. activation time, the correlation coefficient was 0.993 for the curved line but 0.852 for a straight line. Since the data fit the curved line plot better, Ca\(^{2+}\)-stimulated autophosphorylation of C\(\text{CaMK}\) by an intermolecular catalysis is suggested. However, these data do not eliminate the possibility that an intra-molecular catalysis is also involved in the activation by Ca\(^{2+}\).

Ca\(^{2+}\) and Ca\(^{2+}/\text{CaM}\) mediated protein phosphorylation could be considered as conversion of a digital signal (calcium signal) into an analog signal (phosphorylation signal) in the information transfer process. The molecular understanding of this signal transduction will be helpful in studying the amplitude modulation (AM) and frequency modulation (FM) of Ca\(^{2+}\) signals (37,38,39). In plants conversion of Ca\(^{2+}\) signal into a phosphorylation signal is mediated by the Ca\(^{2+}\)-dependent protein kinases and Ca\(^{2+}/\text{CaM}\)-dependent protein kinases. Signal dependent autophosphorylation of a specific amino acid residue has been implicated in the regulation of the catalytic activities of a number of
proteins from animal systems such as p21 (40) and CaMK II (3,41,42). Molecular mechanisms of the second messenger-induced modifications of plant protein kinases are not well understood. Identification of the specific amino acid residue phosphorylated in response to second messenger Ca\(^{2+}\) is an important step in understanding the precise mechanism of Ca\(^{2+}\)/CaM-mediated protein phosphorylation in plants.
Acknowledgements

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**Abbreviations used**

CCaMK, chimeric calcium calmodulin dependent protein kinase; CDPK, calcium dependent protein kinase; CRK, CDPK related protein kinase; CaMK I, calcium/calmodulin dependent protein kinase 1; CaM, calmodulin; GS peptide, glycogen synthase peptide, IMAC, immobilized metal ion chelate affinity chromatography; 4HCCA, α-cyano-4-hydroxycinnamic acid; MALDI-TOF, matrix assisted laser desorption ionization time of flight; PSD, post source decay; SAP, stimulated autophosphorylation.
References


Figure Legends

Figure 1 a, b, c. MALDI mass spectra of IMAC derived phosphopeptides from the tryptic digest of the autophosphorylated CCaMK. 1a and 1b: MALDI-TOF mass spectrum of phosphopeptides showing a major peak (m/z 2167.7) corresponding to the peptide Q253-K269 containing phosphorylated T267. See Table I for mass comparisons. The IMAC fraction obtained using 0.1% ammonium acetate pH 8.0 was used as a control. 1c: PSD spectrum of Q253-K269 showing the metastable loss of H$_3$PO$_4$ (from the loss of 98 Da). The spectrum was externally calibrated by standard peptide mixtures.

Figure 2: Autophosphorylation analysis of wild-type and mutant CCaMK. The wild type and mutant T267A were tested for Ca$^{2+}$-stimulated autophosphorylation as described. See Table II for values. Ca$^{2+}$-stimulation is represented as SAP index, (Stimulated Autophosphorylation) which is the ratio of the amount of $^{32}$[P]PO$_4$ incorporation in the presence of calcium to that in the absence of calcium.

Figure 3: $^{35}$S-labeled CaM binding to the wild type and T267A mutant CCaMK. Fold increase in binding of the $^{35}$S-labeled CaM to the wild type, mutant T267A and the peptide P(257-340) is represented. $^{35}$S-labeled CaM-binding in the presence of calcium to the phosphorylated form of kinase or peptide is compared to the unphosphorylated form. The mean and standard error of minimum four independent measurements is
shown in each bar.

**Figure 4. Ca\(^{2+}/CaM\)-stimulation of Kinase activity of the wild type and T267A mutant.**

GS peptide was used as a substrate to study the kinase activity of the wild-type and mutant CCaMK. The substrate phosphorylation of the wild-type (WT) and T267A in the presence of Ca\(^{2+}/CaM\) is compared to its absence. The mean and standard error of minimum six independent-measurements is shown in each bar.

**Figure 5. Ca\(^{2+}/Calmodulin\) activation of the substrate phosphorylation of T267A.**

Histone IIAS was used as a substrate in the phosphorylation experiments. Solid line represents Histone II AS phosphorylation by T267A in the presence of calcium (0.2 mM Ca\(^{2+}\)) and the dotted line, in the absence of calcium (2.5 mM EGTA) and increasing amounts of calmodulin at 30\(^{\circ}\)C for 10 min as described under "Experimental Procedures".

**Figure 6. Alignment of CaM Kinase sequences:** The amino acid sequences of the catalytic core and CaM-binding regions of the rat CaMK I (17), mouse CaMK II \(\alpha\)-sub unit (18) and mouse CaMK IV (19) are shown with the corresponding amino acid sequences of plant CCaMK (9,10). The conserved regions are shaded using BOXSHADE. The divergent N- and C-terminal regions of the enzymes are not shown. The conserved T267 of CCaMK is marked with asterisk.
Figure 7. Homology model of kinase and calmodulin binding domain of CCaMK based on the structure of CaM kinase 1. The model was generated using MODELLER program using the coordinates of crystal structure of CaM kinase 1 (PDB file 1A06). T267 amino acid residue identified as Ca^{2+} -stimulated autophosphorylation site is shown in magenta. The CaM-binding and autoinhibitory region are labeled based on previous works (12,36). The catalytic site residues are labeled based on the protein kinase classification (32,33). The peptide identified (Q253-K269) from MALDI-TOF MS is labeled in yellow.

Figure 8. Concentration dependence of Ca^{2+}-stimulated CCaMK autophosphorylation. CCaMK at the indicated concentrations was subjected to Ca^{2+}-stimulated autophosphorylation at 30 °C for 10 minutes (see “Experimental Procedures”). Solid triangles, [^{32}P]PO_4 incorporation (autophosphorylation) in the presence of 0.2 mM Ca^{2+} and the solid line represents fit to the data.
<table>
<thead>
<tr>
<th>Amino acid residues of phosphopeptides</th>
<th>Mass (m/z) (theoretical)</th>
<th>Mass (m/z) (observed)</th>
<th>Difference (theo.-obs.)</th>
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</thead>
<tbody>
<tr>
<td>QQRILAGDFSFEEEHTpWK</td>
<td>2172.29</td>
<td>2167.73</td>
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<tr>
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<td>QQRILAGDFSFEEEHTpWKITSSAKDLISSLLSVDPYK</td>
<td>4292.71</td>
<td>4286.61</td>
<td>6.1</td>
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</table>
Table II

**Dependence of autophosphorylation on Ca$^{2+}$ by CCaMK and T267A:**
Autophosphorylation was assayed as described under the "Experimental Procedures". The cpm shown are the average and standard deviation of six gel slice measurements for each sample. Counts cannot be compared between samples, because the amount of protein assayed is not same for each sample. SAP (Stimulated Autophosphorylation) index is the ratio of $[^{32}\text{P}]\text{PO}_4$ incorporation in the presence of calcium (0.2 mM Ca$^{2+}$) to that in the absence of calcium (2.5 mM EGTA). The variance of SAP index was calculated as described in ref. 43.

<table>
<thead>
<tr>
<th>Protein kinase</th>
<th>+ Ca$^{2+}$</th>
<th>- Ca$^{2+}$</th>
<th>SAP index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3083 ± 44</td>
<td>604 ± 35</td>
<td>5.1 ± 0.2</td>
</tr>
<tr>
<td>T267A</td>
<td>519 ± 12</td>
<td>509 ± 16</td>
<td>1.0 ± 0.2</td>
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</table>
Table III

Autophosphorylation sites of animal and plant Calcium/Calmodulin dependent Protein Kinases

<table>
<thead>
<tr>
<th>Protein Kinase</th>
<th>Organism</th>
<th>Phosphorylation Site</th>
<th>Comments</th>
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<tr>
<td>Calcium/calmodulin dependent protein kinase I</td>
<td>Mouse</td>
<td>T167</td>
<td>Ca$^{2+}$/CaM-dependent Activation loop phosphorylation, Ref:17,27,28</td>
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<tr>
<td>Calcium/calmodulin dependent protein kinase II</td>
<td>Mouse</td>
<td>T286</td>
<td>Ca$^{2+}$/CaM-dependent Ref: 15,29</td>
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<tr>
<td>Calcium/calmodulin dependent protein kinase IV</td>
<td>Mouse</td>
<td>S437</td>
<td>Ca$^{2+}$/CaM-dependent Ref: 30,31</td>
</tr>
<tr>
<td>Chimeric Calcium/calmodulin dependent protein kinase</td>
<td>Lily</td>
<td>T267</td>
<td>Ca$^{2+}$-dependent, CaM independent. Ref: this manuscript</td>
</tr>
</tbody>
</table>
Fold increase in 35 S-CaM binding

<table>
<thead>
<tr>
<th></th>
<th>T267A</th>
<th>P(257-340)</th>
<th>WT</th>
</tr>
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<tbody>
<tr>
<td>Value</td>
<td>1</td>
<td>4.5</td>
<td>7.3</td>
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Calcium-stimulated autophosphorylation site of plant chimeric calcium/calmodulin dependent protein kinase
Puthanveetil V. Sathyanarayanan, William F. Siems, Jeffrey P. Jones and B. W. Poovaiah
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