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Molecular and Biochemical Analysis of Calmodulin Interactions with the Calmodulin-Binding Domain of Plant Glutamate Decarboxylase[1]

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We previously provided what to our knowledge is the first evidence that plant glutamate decarboxylase (GAD) is a calmodulin (CaM)-binding protein. Here, we studied the GAD CaM-binding domain in detail. A synthetic peptide of 26 amino acids corresponding to this domain forms a stable complex with Ca^{2+}/CaM with a 1:1 stoichiometry, and amino acid substitutions suggest that tryptophan-485 has an indispensable role in CaM binding. Chemical cross-linking revealed specific CaM/GAD interactions even in the absence of Ca^{2+}. However, increasing KCl concentrations or deletion of two carboxy-terminal lysines abolished these interactions but had a mild effect on CaM/GAD interactions in the presence of Ca^{2+}. We conclude that in the presence of Ca^{2+}-hydrophobic interactions involving tryptophan-485 and electrostatic interactions involving the carboxy-terminal lysines mediate CaM/GAD complex formation. By contrast, in the absence of Ca^{2+}, CaM/GAD interactions are essentially electrostatic and involve the carboxy-terminal lysines. In addition, a tryptophan residue and carboxy-terminal lysines are present in the CaM-binding domain of an Arabidopsis GAD. Finally, we demonstrate that petunia GAD activity is stimulated in vitro by Ca^{2+}/CaM. Our study provides a molecular basis for Ca^{2+}-dependent CaM/GAD interactions and suggests the possible occurrence of Ca^{2+}-independent CaM/GAD interactions.

Understanding signal transduction pathways has become one of the foremost areas of plant biology research, and in particular, considerable progress has been made during the last years in understanding the role of Ca^{2+} as a second messenger (Trewavas and Gilroy, 1991; Roberts and Harmon, 1992; Poovaiah and Reddy, 1993). An increase in the level of cytosolic Ca^{2+} is one of the events involved in the transduction of environmental signals such as light (Shacklock et al., 1992; review by Bowler and Chua, 1994), cold, and touch (Knight et al., 1991, 1992), as well as responses mediated by phytohormones such as GA_3 (Gilroy and Jones, 1992) and ABA (McAinsh et al., 1990). Transient elevations in cytosolic Ca^{2+} levels are transmitted through Ca^{2+}-modulated proteins (Roberts and Harmon, 1992), of which CaM is best characterized. CaM contains four Ca^{2+}-binding sites and is highly conserved among plants and animals. Upon the binding of Ca^{2+}, CaM undergoes conformational changes, which in turn transmit the Ca^{2+} signal by binding to and activating numerous target proteins (Means, 1988). Recent atomic resolution-structure analysis of CaM bound to target peptides is beginning to reveal what the binding looks like and how it differs from one target to another (reviewed by Clore et al., 1993; Torok and Whitaker, 1994).

Several CaM-target proteins have been characterized in animals. These include protein kinases (Colbran and Soderling, 1991), protein phosphatases (Guerini et al., 1991), Ca^{2+} pumps (Hofmann et al., 1994), structural proteins (Hartwig et al., 1992), transcription factors (Corneliussen et al., 1994), and others. In contrast, little is known about the number, localization, identity, and structure of CaM-target proteins in plants (Ling and Assmann, 1992; Baum et al., 1993; Watillon et al., 1993; Hsieh and Roux, 1994; Lu and Harrington, 1994). The lack of information concerning plant CaM-target proteins has been a major obstacle in elucidating Ca^{2+}-mediated signal transduction mechanisms in plants.

We have developed and used molecular procedures to clone cDNAs encoding CaM-binding proteins (Baum et al., 1993) with the idea that such proteins may play a key role in the transduction of environmental stimuli into cellular processes in plants. Using this approach we isolated a cDNA encoding a Ca^{2+}-dependent CaM-binding GAD. Previous studies have shown that this enzyme is rapidly activated in response to a variety of environmental stresses (Streeter and Thompson, 1972a, 1972b; Wallace et al., 1984; Rhodes et al., 1986; Randall et al., 1990). Our findings suggest that Ca^{2+} signaling via CaM may be involved in regulating synthesis of γ-aminobutyric acid in stress situations (Baum et al., 1993). The available tools resulting

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Abbreviations: CaM, calmodulin; CaMBD, CaM-binding domain; CaMKII, CaM-dependent protein kinase II; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; GAD, L-glutamate decarboxylase; GST, glutathione S-transferase; PBSED, phosphate-buffered saline + EDTA + DTT; smMLCK, smooth muscle myosin light-chain kinase.

from our molecular approach provide an excellent opportunity to conduct a multidisciplinary study of the involvement of Ca^{2+} and CaM in plant responses to stresses. Here, we dissected the CaMBD of the petunia GAD and revealed amino acid residues essential for CaM/GAD interactions.

MATERIALS AND METHODS

CaM/Peptide Complex Detection on Nondenaturing PAGE

Samples containing 240 pmol (4 μg) of CaM and different amounts of a HPLC-purified synthetic peptide (provided by Prof. M. Fridkin, Department of Organic Chemistry, Weizmann Institute of Science) in 100 mM Tris-HCl, pH 7.2, and either 0.1 mM $CaCl_2$ or 2 mM EGTA in a total volume of 30 μL were incubated for 1 h at room temperature. A one-half volume of 50% glycerol with tracer bromphenol blue was added and samples were then loaded onto nondenaturing slab gels (1.5 mm thickness) containing 12.5% acrylamide in 375 mM Tris-HCl, pH 8.8, and 0.1 mM $CaCl_2$ or 2 mM EGTA and electrophoresed at a constant current of 25 mA in running buffer consisting of 25 mM Tris-HCl and 192 mM Gly, pH 8.3, with 0.1 mM $CaCl_2$ or 2 mM EGTA. The gels were stained with Coomassie brilliant blue.

Extraction and Purification of GST-Fusion Proteins

Overnight cultures of *Escherichia coli* (XL1-Blue strain; Stratagene) harboring plasmid pGEX-3X (Smith and Johnson, 1988) (Pharmacia), which included different DNA inserts derived from the GAD cDNA, were diluted 1:10 in 800 mL of M9 plus Bactotrypton (Sambrook et al., 1989). Cultures were grown with vigorous agitation at 37°C until the A_{600} reached 0.2. Isopropyl β-D-thiogalactopyranoside was added (1 mM final concentration), and cultures were grown for another 2 h. Cells were pelleted at 4000*g* and resuspended at 1:100 of the original volume with cold PBSED extraction buffer (16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT) and protease inhibitors (1 mM PMSF, 7.5 μg mL^{-1} antipain, 7.5 μg mL^{-1} leupeptin). Freshly prepared lysozyme was added to a final concentration of 200 μg mL^{-1} for 10 min. Then $MgCl_2$ (3 mM) and DNase I (50 μg mL^{-1}) were added, and the mixture was kept on ice for another 20 min. After Triton X-100 was added to 1% (v/v), the lysate was centrifuged at 30,000 rpm in a Beckman Ti75 rotor for 30 min. The supernatant containing the soluble fusion protein was collected and loaded on a GSH-agarose column (Sigma) pre-equilibrated with PBSED plus 1% Triton X-100. The column was washed with 10 column volumes of PBSED plus 1% Triton X-100, and the fusion protein was eluted by the addition of a solution containing 5 mM reduced GSH in 50 mM Tris-HCl, pH 7.6.

^{35}S-Recombinant CaM Binding to Proteins on Blots

Proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose, and treated with ^{35}S-recombinant CaM as described by Baum et al. (1993). Following autoradiography of blots, immunodetection of proteins on the same blots was performed as follows. Nitrocellulose membranes were washed for 30 min in PBS containing 0.05% Tween 20 and 1% nonfat milk. All additional immunostaining steps were performed as previously described (Baum et al., 1993).

CaM-Agarose Affinity Chromatography of GST-Fusion Proteins

Samples of purified GST-fusion proteins containing $CaCl_2$ (10 mM) and NaCl (150 mM) were loaded onto CaM-agarose (Sigma) columns pre-equilibrated with CaM-binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM $CaCl_2$). Columns were washed with 10 column volumes of CaM-binding buffer, and CaM-binding proteins were eluted by the addition of a solution containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2 mM EGTA. The columns contained about 300 μg of bound CaM, whereas the maximal amount of loaded protein was 10 μg.

In Vitro Cross-Linking of CaM to Proteins

Samples with purified fusion proteins or purified peptides were dialyzed at 4°C versus 50 mM Hepes, pH 7.5, for 2 h. The dialyzed proteins were incubated with bovine CaM in 50 mM Hepes, 100 mM KCl (unless indicated otherwise), and 1 mM Ca^{2+} or 2 mM EGTA (wherever indicated) for 1 h at 22°C. Cross-linking was performed by adding EDC (Pierce) and sulfo-*N*-hydroxysulfosuccinimide (Pierce) to half of the final concentration indicated. After 10 min of incubation at 22°C, EDC and *N*-hydroxysulfosuccinimide were added again to their final concentrations. After incubation for another 20 min, the reaction was stopped by the addition of 3× SDS-PAGE loading buffer and was boiled for 3 min. The products of the cross-linking were analyzed by standard SDS-PAGE (Baum et al., 1993) followed by Coomassie brilliant blue staining.

Site-Directed Mutagenesis

A DNA fragment corresponding to the coding region of the GAD-CaMBD (amino acids 469–500; nucleotides 1475–1574; Baum et al., 1993) was amplified by PCR with oligonucleotides containing a *Bam*HI site on the sense oligonucleotide and an *Eco*RI site on the antisense oligonucleotide as described by Baum et al. (1993). The *Eco*RI-*Bam*HI fragment (320 bp) was subcloned into M13 mp19 phage DNA. Oligonucleotide-directed site-specific mutagenesis was carried out using the Bio-Rad Muta-Gene kit as follows. First, Trp^{485} was substituted with Arg by priming with the antisense oligonucleotide 5′-AACAAACTTCTTGCGCG-CAGTTATCATCTC-3′. Putative mutants were identified by the newly created *Bss*HII site (GCGCGC). Other substitutions of Trp^{485} were created with similar oligonucleotides, except that they included the following relevant codons (in the antisense oligonucleotide): Leu (GAG), Phe (AAA), Tyr (ATA), Ala (CGT), Cys (ACG), and Glu (CTC). In addition, these mutants were created on the Arg^{485}

mutant-DNA template and were identified by the disappearance of the *Bss*HII site. Phe^{488} was substituted with Ala by priming with the antisense oligonucleotide 5′-CTTTTCTTCAACTGCCTTCTTCCACGCAGTTATCAT-3′. Following mutagenesis, DNA fragments corresponding to the GAD-CaMBD mutants were subjected to PCR amplification with the universal reverse and forward M13 primers. The amplified fragments were digested with *Bam*HI and *Eco*RI and subcloned into the *Bam*HI-*Eco*RI sites of a pGEX-3X vector. This created in-frame fusions of the GST-coding sequence of the vector with the coding region of the GAD-CaMBD mutants. The nucleotide sequences of all cloned fragments derived from mutagenesis and PCR amplification were determined after cloning into the pGEX-3X vector by using as primers oligonucleotides designed for sequencing from both sides of the pGEX-3X-cloning sites.

Construction of DNA Templates Coding for Truncated GAD Proteins

Templates coding for carboxy terminus-deleted GAD proteins were produced by PCR amplification of the GAD-coding sequence from the original cDNA (Baum et al., 1993) with GAD-specific oligonucleotides containing appropriate restriction sites for cloning into a pET12C expression vector (Studier et al., 1990; Novagen, Madison, WI). The GAD-CΔ11 amplified fragment contained an *Nde*I site at the 5′ end and an *Eco*RI site at the 3′ end. The GAD-CΔ27 amplified fragment had an *Nde*I site at its 5′ end and an *Sal*I at its 3′ end. Expression of GAD and truncated GAD proteins was performed in *E. coli* strain BL21(DE3) pLysS according to the method of Studier et al. (1990). Templates for amino and carboxy termini-deleted forms of the GAD-CaMBD were produced by PCR amplification of the corresponding GAD-CaMBD-coding region, with specific oligonucleotides as primers. The sense primers used for PCR amplification contained a *Bam*HI site, and the antisense primers generally contained an *Eco*RI site. Only for GST-CaMBD, GST-CaMBD-CΔ11, and GST-CaMBD-NΔ5 (Baum et al., 1993) was a blunt end used for cloning at the 3′ site. The PCR fragments were digested with *Eco*RI and *Bam*HI and cloned into the *Bam*HI-*Eco*RI sites of a pGEX-3X vector, thus creating an in-frame fusion of the coding sequence for GSH S-transferase on the pGEX 3X vector and the deleted forms of GAD-CaMBD. The nucleotide sequences of all cloned fragments derived by PCR amplification were determined after cloning into the pGEX-3X vector, using oligonucleotides designed for sequencing from both sides of the pGEX-3X cloning sites as primers.

Extraction of Petunia Petal GAD and Determination of Its Specific Activity

GAD was partially purified from petunia petals and GAD activity was determined essentially as described by Ling et al. (1994), except that 0.2 mM EDTA was added to chelate traces of Ca^{2+} in treatments that did not contain Ca^{2+}.

RESULTS

The Petunia GAD Contains a Single CaMBD

We have shown that the 32-amino acid carboxy end of the petunia GAD functions as a CaMBD when fused to a protein that otherwise does not bind to CaM (Baum et al., 1993). Before analyzing in detail the properties of this GAD-CaMBD, we determined whether this is the only CaMBD in the petunia GAD. The complete recombinant GAD and two truncated forms lacking 27 or 11 amino acids from the carboxy end (GAD-CΔ27 and GAD-CΔ11, respectively) were produced in *E. coli*. Total *E. coli* proteins were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane. Binding of ^{35}S-recombinant CaM to the recombinant proteins (Fig. 1) showed that the complete GAD, but neither of the truncated proteins, bound CaM. Immunodetection of GAD confirmed the presence of similar amounts of the complete GAD and the two truncated mutants on the blot. Thus, in the absence of the carboxy-end domain, CaM cannot bind to GAD. The anti-GAD antibodies also detected a major breakdown product of the recombinant GAD that did not bind to CaM.

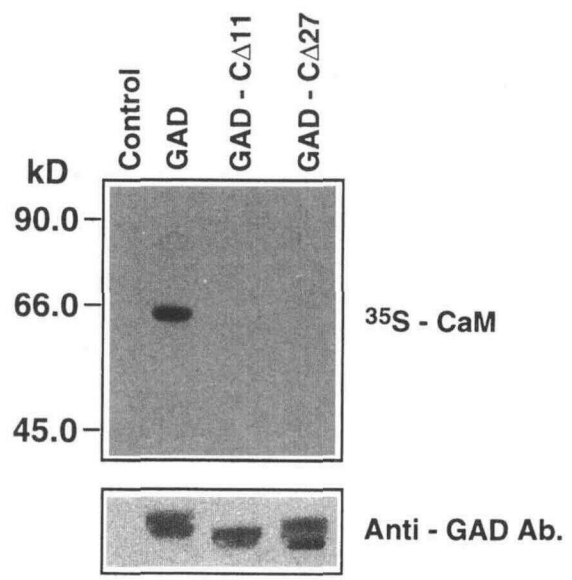


Figure 1. The petunia GAD contains a single CaMBD. The full-length recombinant GAD and truncated mutants lacking 27 or 11 amino acids from the carboxy end were prepared as described in "Materials and Methods." Equal amounts (20 μg) of total *E. coli* protein extracts containing either the full-length or truncated GAD proteins were separated on SDS-PAGE, electrotransferred to nitrocellulose membranes, and incubated with ^{35}S-recombinant CaM (top). An extract with equal amounts of proteins from *E. coli* containing the expression vector without an insert was used as a negative control. Following autoradiography, the blot was incubated with polyclonal antibodies (Ab.) raised against the recombinant GAD (bottom). Protein-antibody complexes were detected with an ECL kit (Amersham). The lane marked kD contains molecular mass protein markers.

A Synthetic Peptide Corresponding to GAD Amino Acids 470 to 495 Binds to Ca^{2+}/CaM

To determine whether a peptide corresponding to the GAD carboxy end (Baum et al., 1993) binds to CaM independently of any other protein sequences and to quantify the interaction between the binding site and Ca^{2+}/CaM, an HPLC-purified synthetic peptide corresponding to GAD amino acids 470 to 495 (Baum et al., 1993) was incubated with bovine CaM, and complex formation was assessed by nondenaturing PAGE (Cox et al., 1985) in the presence or absence of Ca^{2+}. The results (Fig. 2) show that the peptide is capable of forming a stable complex with CaM in the presence of Ca^{2+} but not in the absence of Ca^{2+} (i.e. in the presence of 2 mM EGTA). Several ratios of peptide to CaM were used. In the absence of the peptide, there was a single band reflecting pure CaM. As peptide was added, another band of lower mobility appeared, representing the peptide/CaM complex. When the ratio of peptide to CaM was equal, almost all of the CaM disappeared, and the intensity of the peptide/CaM complex increased. At a peptide to CaM molar ratio of 1.5, no free CaM was detected. At still higher ratios (up to 2.5), no new band appeared on the gel, nor did the peptide/CaM complex band change its intensity, suggesting that multivalent complexes were absent. These observations indicate that the peptide binds Ca^{2+}/CaM with a 1:1 stoichiometry.

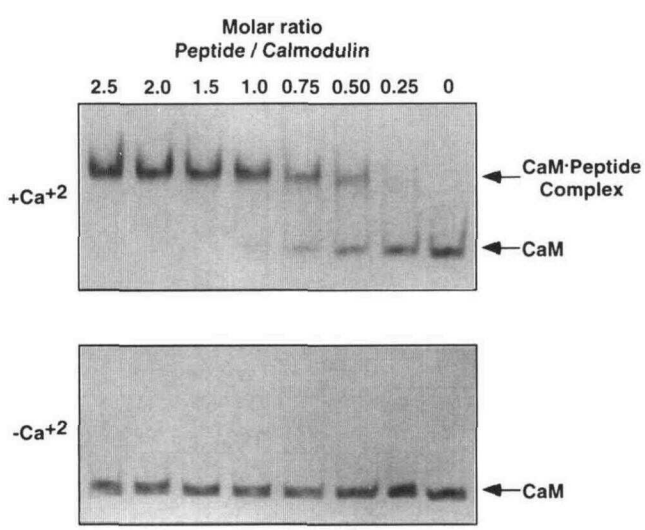


Figure 2. A purified synthetic peptide forms a stable complex with CaM. A peptide corresponding to petunia GAD amino acids 470 to 495 (Baum et al., 1993) was prepared as described in "Materials and Methods." Complex formation between CaM and the peptide was assayed in the presence of 0.1 mM $CaCl_2$ (top) or absence of $CaCl_2$ (including 2 mM EGTA; bottom). Increasing amounts of the peptide (peptide/CaM molar ratios indicated) were incubated with 240 pmol of bovine CaM, and then samples were separated by nondenaturing PAGE. Arrows indicate the positions of the free CaM and the CaM/peptide complex. The free peptide is not apparent because it had run out of the gels by the time electrophoresis was stopped.

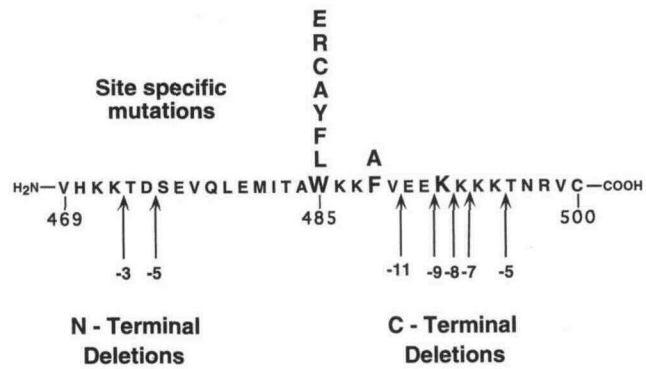


Figure 3. Schematic presentation of amino acid substitutions and deletions in the GAD-CaMBD (amino acids 469–500; Baum et al., 1993). Amino acid substitutions resulting from site-specific mutations are indicated above the wild-type amino acid sequence. Deletion sites are indicated by arrows.

Trp^{485} Is Essential for Ca^{2+}-Dependent CaM Binding

The identification of the carboxy terminus as the unique CaM-binding site in GAD and the fact that it functions as a purified peptide independently of any other GAD sequences allowed us to delimit the CaMBD more precisely, to determine which amino acid residues are important for CaM binding, and to elucidate the nature of the apparent CaM/GAD interactions.

Previously, we noted that the basic amphiphilic motif $Trp^{485}Lys^{486}Lys^{487}$ of petunia GAD is present in CaMBDs of some proteins from other organisms (Baum et al., 1993). In these proteins, the Trp residue plays a crucial role in CaM binding (Ikura et al., 1992; Clore et al., 1993) through interactions with a hydrophobic pocket in Ca^{2+}-bound CaM (O'Neil and DeGrado, 1990; Clore et al., 1993; Meador et al., 1993). The Lys residues interact with negatively charged residues of the acidic CaM (Clore et al., 1993). However, because CaMBDs are not sequence conserved (O'Neil and DeGrado, 1990) and because no CaMBD of a plant protein has ever been analyzed in detail, we introduced several amino acid substitutions in the position of Trp^{485} to assess the importance of Trp^{485} in binding CaM. These amino acid substitutions, as well as other mutations and deletions within the carboxy-end domain of GAD discussed below, are presented in Figure 3.

Wild-type and mutant domains with amino acid substitutions of Trp^{485} were then analyzed for their ability to bind CaM as fusion proteins with the coding sequence of GST that itself does not bind CaM (Baum et al., 1993). Binding of wild-type and mutant domains to CaM was first tested by ^{35}S-recombinant CaM overlay on blots in the presence of Ca^{2+} (Fig. 4). This analysis showed that no residue of the seven that were used to replace Trp can confer CaM binding as effectively as the wild-type domain. The only two substitutions that showed some binding to CaM on the blot were Leu and to a lesser extent Phe. By contrast, replacement of the only other aromatic residue in the domain (Phe^{488}) with Ala had a negligible effect on CaM binding (Fig. 4). Furthermore, an HPLC-purified synthetic peptide of 26 amino acids similar to that used in

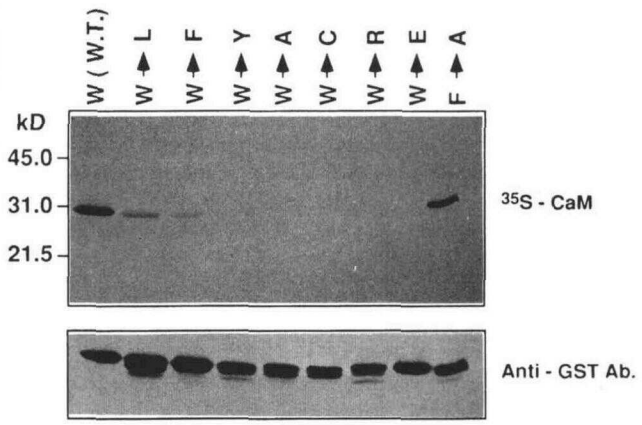


Figure 4. CaM binding to wild type and mutants of the GAD-CaMBD (cf. Fig. 3). Equal amounts (3 μg) of purified wild-type and mutant GST-CaMBD fusion proteins (prepared and purified as described in "Materials and Methods") were separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane, and incubated with ^{35}S-recombinant CaM. Following autoradiography, the blot was incubated with anti-GST antibodies (Ab.; bottom) and protein-antibody complexes were detected with an ECL kit (Amersham). The position of the wild-type lane containing the Trp residue [W (W.T.)] and the various mutants containing substitutions of Trp^{485} or Phe^{488} are indicated above the autoradiogram using the one-letter code for amino acids (top). The lane marked kD contained molecular mass protein markers.

Figure 2, but with a substitution of Trp to Arg, did not form a complex with CaM (not shown). These results suggest that Trp^{485} has a specific and crucial role in CaM binding to GAD in the presence of Ca^{2+}. Similar relative affinities were found by binding to CaM-agarose columns (Fig. 5). With the substitution of Trp^{485} by Leu or Phe, all loaded proteins bound to the column, although Leu and Phe mutants were eluted slightly earlier. Substitution by Tyr, Ala, and Cys or by Arg and Glu resulted, respectively, in recovery of some or all of the loaded proteins in the effluent (Fig. 5).

The overall picture emerging is that any charged-residue substitution of Trp^{485} abolished CaM binding. Even a substitution to a positively charged residue that may increase the electrostatic interactions with acidic CaM residues failed to bind CaM. This indicates that hydrophobic interactions are essential to Ca^{2+}-dependent CaM/GAD binding, consistent with the suggested cardinal role of hydrophobic interactions in Ca^{2+}-dependent CaM binding to other target proteins (O'Neil and DeGrado, 1990; Clore et al., 1993). Indeed, the two most potent replacements of Trp in our study were Leu and Phe, which possess relatively large hydrophobic side chains, as opposed to either Tyr, an aromatic residue with a polar group, or Ala, a residue with a small hydrophobic surface, both of which conferred weaker binding. Interestingly, Phe has a larger hydrophobic surface than Leu and Trp but binds less strongly to CaM; this suggests that other properties of Trp^{485} are also important. For example, Trp^{485} may form hydrogen bonds with adjacent residues, whereas Phe cannot. Recent x-ray structure analysis of CaM complexes with peptides from CaMKII and from smMLCK showed that the same hydrophobic pocket in the carboxy-terminal domain of CaM is occupied by Leu and Trp residues, respectively (Meador et al., 1993). Thus, the observation that Leu can replace Trp in conferring CaM binding is consistent with these studies and suggests some common features between the hydrophobic interactions of CaM and GAD and the two mammalian peptides mentioned above.

Electrostatic CaM/GAD Interactions in the Presence and in the Absence of Ca^{2+}

It is well documented that CaM-target enzymes are activated in response to the binding of Ca^{2+} to CaM and the concomitant formation of a stable Ca^{2+}/CaM-enzyme complex. A few of these complexes have been subjected to detailed structural analysis by x-ray crystallography and NMR (reviewed by Clore et al., 1993; Torok and Whitaker, 1994). However, based on thermodynamic considerations Klee et al. (1986) suggested that for rapid activation of Ca^{2+}/CaM-regulated enzymes in vivo, CaM-enzyme complexes must exist in the absence of a Ca^{2+} signal because otherwise Ca^{2+}/CaM-enzyme complex formation will rely

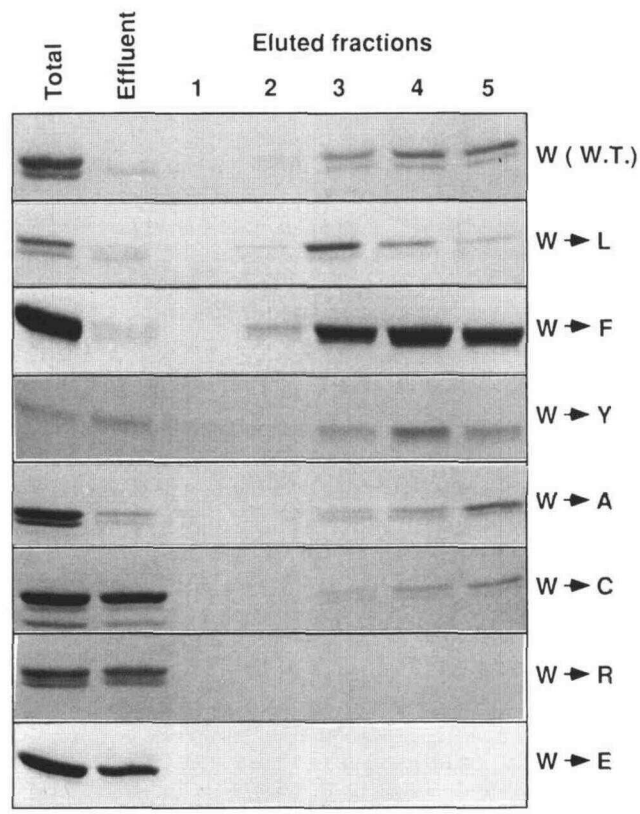


Figure 5. Binding of wild-type (W.T.) CaMBD and Trp^{485}-substitution mutants to CaM-agarose columns. Samples (5–10 μg) of purified GST-CaMBD fusion proteins (wild type and mutants as indicated) were passed through CaM-agarose columns as described in "Materials and Methods." Samples of the total protein loaded onto the column, the effluent fraction, and equal volumes of the first to fifth EGTA-eluted fractions (1–5, respectively) were separated on SDS-PAGE (12.5%). The Coomassie brilliant blue-stained gel is presented.

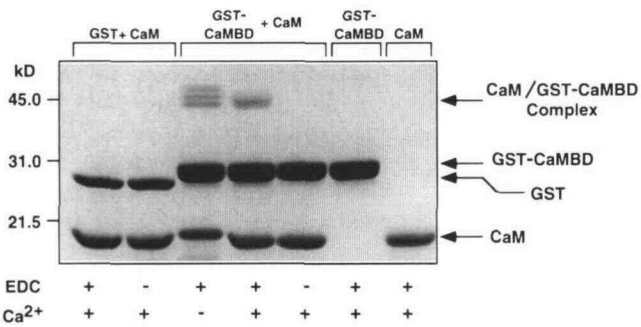


Figure 6. Cross-linking of CaM to the GAD-CaMBD by EDC in the presence or absence of Ca^{2+}. Reactions included 18 μM of each of the following proteins as indicated above the lanes: GST + CaM, GST-CaMBD + CaM, GST-CaMBD alone, or CaM alone, in the presence of 1 mM $CaCl_2$ (+) or in the absence of $CaCl_2$ (with 2 mM EGTA) (−) and with (+) or without (−) 1 mM EDC, as indicated. Reaction products were separated by SDS-PAGE (12.5%) and stained with Coomassie brilliant blue. The positions of CaM, GST, GST-CaMBD, and the cross-linked CaM/GST-CaMBD complexes are indicated by arrows.

on macromolecule diffusion, which is relatively slow. Indeed, recent studies of CaM-binding proteins suggest that CaM-target interactions may occur even in the absence of Ca^{2+} (Itakura and Iio, 1992). In addition, some CaM-target proteins such as neuromodulin bind to CaM preferentially at low Ca^{2+} concentrations (Alexander et al., 1988), and in yeast, essential calmodulin functions seem to be Ca^{2+} independent (Geiser et al., 1991).

To assess the possible occurrence of CaM/GAD interactions in the absence of Ca^{2+}, we used the zero-length cross-linking chemical reagent EDC as described for studies of other CaM/protein interactions (Giedroc et al., 1983; Minami et al., 1993). We performed cross-linking experiments between the fusion protein GST-CaMBD and CaM in the presence of EDC with or without Ca^{2+} (i.e. including 2 mM EGTA). Following incubation, proteins were separated by SDS-PAGE and covalently formed protein complexes were observed by Coomassie staining of gels (Fig. 6). When CaM was incubated with the GST-CaMBD fusion protein in the presence of EDC, a covalently linked complex was formed. This complex was of the expected molecular mass for a single CaM/GST-CaMBD interaction (about 45 kD), consistent with the 1:1 CaM-peptide stoichiometry found in Figure 2. In addition, CaM/CaMBD interactions in the absence of Ca^{2+} occurred as readily as in the presence of Ca^{2+} (Fig. 6). We tested several controls to confirm the specificity of the interaction between CaM and CaMBD in this assay. First, CaM did not cross-link to GST (lacking the CaMBD). Second, GST-CaMBD alone or CaM alone did not form any complexes. Third, incubation of GST-CaMBD with CaM in the absence of EDC also did not form any stable complex on SDS-PAGE.

The identity of the apparent complexes as CaM/GST-CaMBD was verified by using ^{35}S-recombinant CaM as the substrate for cross-linking and the detection of a radioactive band at the position of the apparent complex and by probing protein blots with antibodies against CaM and GST (not shown). We note that the cross-linked products appear as multiple bands on SDS-PAGE. This is likely due to some intramolecular cross-linking that can affect the mobility of proteins on SDS-PAGE. Moreover, CaM itself migrates differently on SDS-PAGE when different numbers of Ca^{2+} sites within it are occupied (Maune et al., 1992).

To investigate the possible involvement of electrostatic forces in CaM/GAD interactions, we performed cross-linking experiments as described above but with increasing KCl concentration. The results (Fig. 7) show that, when the KCl concentration was increased to 100 mM in the absence of Ca^{2+}, CaM/GAD interactions were drastically reduced, whereas in the presence of Ca^{2+} CaM/GAD interactions were only mildly affected. Moreover, in the presence of Ca^{2+}, CaM/GAD interactions were still apparent even at 500 mM KCl. These results are consistent with previous evidence that in the presence of Ca^{2+} CaM-target interactions are mostly hydrophobic, with electrostatic forces contributing only about 15% to the overall interactions (O'Neil and DeGrado, 1990). Conversely, in the absence of Ca^{2+} CaM/GAD interactions are likely to be essentially electrostatic.

The possible occurrence of electrostatic CaM/GAD interactions directed our attention to a unique stretch of Lys residues at the carboxy terminus of the GAD-CaMBD (Lys^{492}–Lys^{495}; Fig. 3). These Lys residues may facilitate electrostatic interactions between GAD and acidic CaM residues in a role similar to that of Lys residues in other CaM-target sites (Clore et al., 1993). To assess this possibility directly, we tested the cross-linking of CaM to carboxy terminus-deleted mutants of the GAD-CaMBD in the

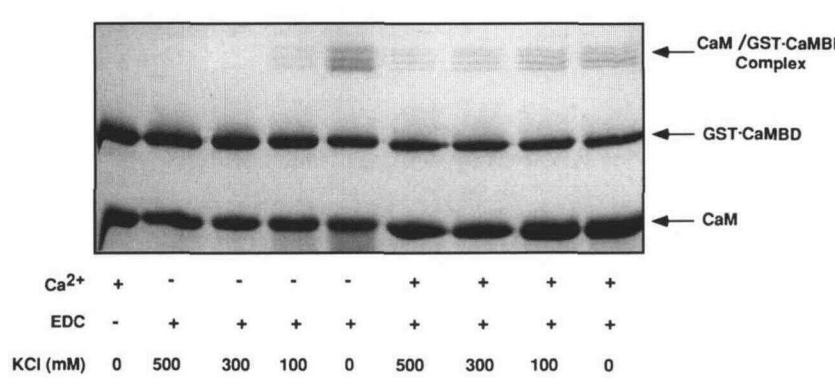


Figure 7. Cross-linking of CaM to GAD-CaMBD at increasing KCl concentrations. Cross-linking was performed in the presence of 1 mM $CaCl_2$ (+), or in the absence of $CaCl_2$ (with 2 mM EGTA) (−) and with (+) or without (−) 1 mM EDC and KCl as indicated. Samples of 5.9 μM GST-CaMBD and 18.7 μM CaM were used in this experiment. Samples were separated by SDS-PAGE (12.5%), and the gel was stained with Coomassie brilliant blue. The positions of CaM, GST-CaMBD, and CaM/GST-CaMBD complexes are indicated by arrows.

presence or absence of Ca^{2+} (Fig. 8). The results revealed a few interesting observations. First, the five amino acids at the very carboxy end (Thr^{496}–Cys^{500}) were not essential for CaM/GAD interactions either in the presence or in the absence of Ca^{2+}. However, removal of two Lys residues from the carboxy end (Lys^{494} and Lys^{495}) abolished cross-linking in the absence of Ca^{2+}. These results indicate that the carboxy-terminal Lys residues are involved in electrostatic interactions between CaM and GAD.

Conversely, in the presence of Ca^{2+}, cross-linking occurred even when 11 amino acids were deleted from the carboxy end. No cross-linking occurred when GST (lacking the CaMBD) was incubated with CaM as a control. However, CaM-overlay assays on blots (in the presence of Ca^{2+}) revealed that removal of Lys residues from the carboxy terminus did reduce the binding affinities to CaM (Fig. 9). Here again, deletion of only 5 amino acids from the carboxy end did not affect CaM binding. Deletion of two Lys residues (CΔ7) had a mild effect on CaM binding (Fig. 9). Any further deletion had obvious effects on CaM binding, such that CΔ8 showed significantly weaker CaM binding. Further deletion of one Lys (CΔ9) reduced CaM binding even more, and when 11 amino acids were deleted no binding of CaM was apparent. This result is also consistent with the observation that removal of the same 11 amino acids from the whole recombinant GAD abolished CaM binding by the overlay assay (cf. Fig. 1).

Because the amino terminus of the petunia GAD-CaMBD contains a stretch of three positively charged residues ($His^{470}Lys^{471}Lys^{472}$), we investigated whether these residues also affect CaM binding in the presence or absence of Ca^{2+}. Deletion of $His^{470}Lys^{471}$ (NΔ3; cf. Fig. 3) had little effect on CaM binding (Fig. 10). Further deletion of two more amino acids including Lys^{472} (NΔ5; cf. Fig. 3) had an obvious effect on CaM binding (Fig. 10). Deletions of these residues, although leaving the carboxy side intact, did not affect cross-linking in the absence of Ca^{2+} (not shown). Thus, although the amino-terminal positively charged residues contribute to CaM binding, under the conditions we used they seem to be less effective than the Lys residues at the carboxy end.

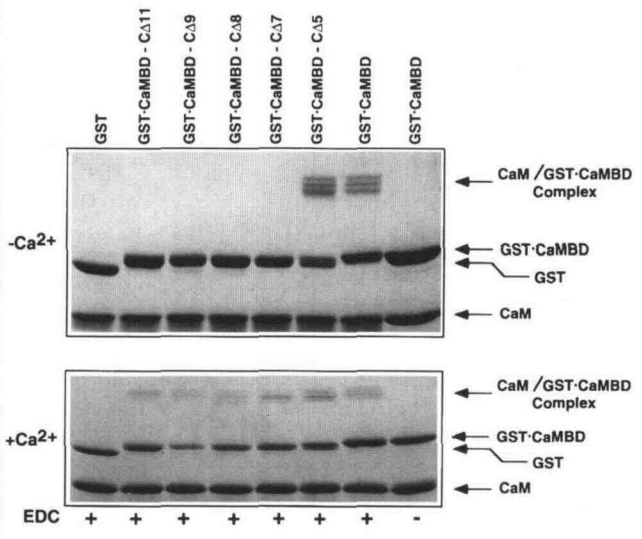


Figure 8. Cross-linking of CaM to carboxy-terminal deletion mutants of GAD-CaMBD (cf. Fig. 3). Reactions were performed in the presence (+) or absence (−) of 1.87 mM EDC and 5.9 μM GST or GST-CaMBD (wild type and deletion mutants as indicated; cf. Fig. 3) with 18.7 μM bovine CaM as indicated, in the absence of Ca^{2+} (with 2 mM EGTA) and no KCl (top) or in the presence of 1 mM $CaCl_2$ and 100 mM KCl (bottom). Cross-linking products were analyzed on SDS-PAGE (12.5%) and stained with Coomassie brilliant blue. The positions of CaM, GST-CaMBD, and CaM/GST-CaMBD complexes are indicated by arrows.

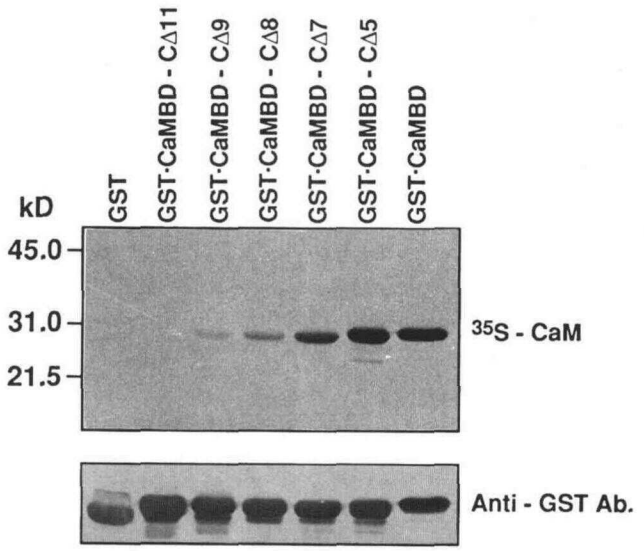


Figure 9. 35S-recombinant CaM binding to GAD-CaMBD carboxy terminus-deleted mutants (cf. Fig. 3). Equal amounts (3 μg) of purified carboxy-terminal deletion mutants of CaMBD (as indicated) fused to GST and GST alone as a negative control were separated on SDS-PAGE (12.5%), electrotransferred to nitrocellulose, and incubated with ^{35}S-recombinant CaM (top). Following autoradiography, the blot was reacted with polyclonal antibodies (Ab.) raised against the GST protein (bottom). Protein-antibody complexes were detected with an ECL kit (Amersham). The lane marked kD contains molecular mass protein markers.

Stimulation of Petunia GAD Activity by Ca^{2+}/CaM

In view of the CaM/GAD interactions discussed above, we determined whether the petunia petal GAD is activated by CaM and whether activation is Ca^{2+} dependent. GAD was partially purified from petunia petals, and activity was measured in the presence or absence of Ca^{2+} and CaM. Table I shows that maximal activation of GAD occurred in the presence of both CaM and Ca^{2+}. The activation of petunia GAD by Ca^{2+}/CaM (276%) was higher than the activation of *Vicia fava* root GAD (212%; Ling et al., 1994). A small increase in GAD activity with Ca^{2+} in the absence of CaM may be attributed to the inability to remove all bound CaM during partial purification of GAD, as determined in similar soybean GAD preparations with monoclonal antibodies against plant CaM (W.A. Snedden and B.J. Shelp, personal communication). The slight activation of GAD by CaM in the absence of Ca^{2+} (in the presence of

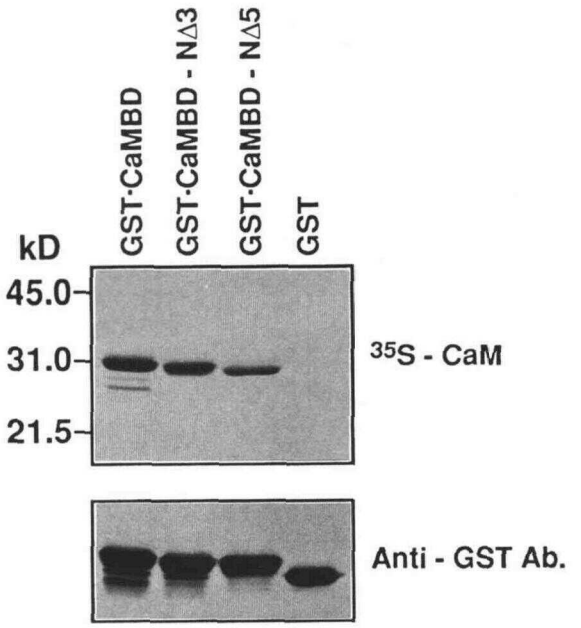


Figure 10. ^{35}S-recombinant CaM binding to amino terminus-deleted mutants of GAD-CaMBD (cf. Fig. 3). Equal amounts (3 μg) of purified amino-terminal deletions of CaMBD (as indicated) fused to GST and GST as a control were separated on SDS-PAGE, electrotransferred to a nitrocellulose membrane, and incubated with ^{35}S-CaM (top). The blot was then reacted with anti-GST antibodies (Ab.; bottom).

0.2 mM EDTA) may be explained by CaM/GAD interactions in the absence of Ca^{2+} as discussed above (cf. Figs. 6–8).

Analysis of the CaMBD of an *Arabidopsis thaliana* GAD

So far, the only GAD-CaMBD that was analyzed at the molecular level was the one from petunia (Baum et al., 1993, and this work). To assess whether GAD from other plants has similar properties with respect to the CaMBD, we determined the complete nucleotide sequence of an *A. thaliana* GAD cDNA (T. Arazi, G. Baum, W.A. Snedden, B.J. Shelp, and H. Fromm, unpublished data) and analyzed the CaM-binding properties of its 30-amino acid carboxy end (amino acids 473–502). A comparison of the amino acid sequences of the carboxy-end domain of Arabidopsis and petunia GAD (amino acids 469–500; Baum et al., 1993) showed only 50% identity, whereas the overall amino acid sequence identity of GAD from Arabidopsis and petunia is about 85% (T. Arazi, G. Baum, W.A. Snedden, B.J. Shelp, and H. Fromm, unpublished data). However, because CaMBDs are generally not sequence conserved (O'Neil and DeGrado, 1990), we determined experimentally whether the Arabidopsis carboxy-end domain can bind to CaM. A DNA fragment containing the coding region of this domain was cloned in frame with the coding sequence of GST, as was done previously with the petunia GAD-CaMBD (Baum et al., 1993). An ^{35}S-CaM overlay shows that the Arabidopsis and petunia carboxy-end domains bind to CaM with similar affinities (Fig. 11A).

The comparison of the amino acid sequences of the CaMBDs of petunia and Arabidopsis GAD (Fig. 11B) shows that the three structural motifs revealed while analyzing the petunia GAD-CaMBD are present also in the Arabidopsis GAD, namely the basic amphiphilic motif TrpLysLys and adjacent residues, the positively charged residues flanking the CaMBD, and the five amino acids at the very carboxy end that do not participate directly in CaM binding. The distribution of charges within the GAD-CaMBD is also interesting. On the carboxy side of the Trp residue (petunia GAD amino acids 486–495, Arabidopsis GAD amino acids 489–497), both petunia and Arabidopsis GAD have an identical net charge of +4 in spite of differences in amino acid residues.

DISCUSSION

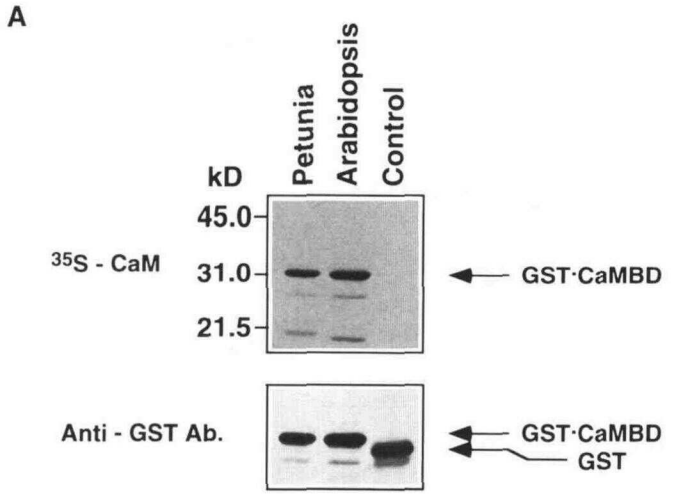
One of the most intriguing questions concerning the interaction of CaM with target proteins is how a phylogenetically conserved protein like CaM can specifically interact with so many different target sites that have little amino acid sequence homology and, occasionally, no obvious predicted structure similarity (O'Neil and DeGrado, 1990). Recent atomic resolution analysis of CaM/peptide complexes provided some answers to this question (reviewed by Clore et al., 1993; Torok and Whitaker, 1994). It appears that the flexibility of the central CaM helix provides a mechanism by which the highly conserved CaM can bind to a wide variety of target enzymes whose CaM-binding sequences display great variability. On the other hand, specificity of binding could be achieved via hydrophobic and electrostatic interactions between specific amino acid residues (Trewhella, 1992).

The recent cloning of the cDNA encoding the petunia CaM-binding GAD (Baum et al., 1993) has enabled detailed characterization of its interactions with CaM, as well as studies of its expression (Baum et al., 1994; Chen et al., 1994). Preliminary analysis of the petunia GAD-CaMBD revealed a TrpLysLys motif (Baum et al., 1993), which is present in some other known CaM targets (Clore et al., 1993). In our present study, we showed that Trp^{485} is indeed an important constituent of the GAD-CaMBD. However, CaM binding was still retained when this Trp residue was replaced by residues with large hydrophobic side chains (Leu and Phe), although these substitutions did not confer CaM binding as effectively as did the Trp-

Table I. *Stimulation of petunia GAD activity by Ca^{2+} and CaM*

GAD was partially purified from petunia petals, and GAD activity was determined as described in "Materials and Methods" without the addition of Ca^{2+} and CaM (control) or with the addition of 500 μM $CaCl_2$ (+ Ca^{2+}), 200 nM CaM (+ CaM), or 500 μM $CaCl_2$ and 200 nM CaM (+ Ca^{2+}/CaM).

Treatment	GAD Specific Activity	
	nmol $min^{-1}mg^{-1}$ protein	*% of control*
Control	103 ± 6	100
+ Ca^{2+}	165 ± 5	160
+ CaM	152 ± 16	148
+ Ca^{2+} and CaM	276 ± 26	269



B

473 - V K K S D I D K Q R D I I T G W K K F V A D . R K K T S G I C - 502 *Arabidopsis*
470 - H K K T D S E V Q L E M I T A W K K F V E E K K K K T N R V C - 500 *Petunia*

Figure 11. Analysis of the CaMBD of an *A. thaliana* GAD. A, The 30-amino acid carboxy-terminal domain of Arabidopsis GAD (GenBank accession number U10034; T. Arazi, G. Baum, W.A. Snedden, B.J. Shelp, and H. Fromm, unpublished data) was fused in frame to the coding sequence of GST as described in "Materials and Methods." Equal amounts (20 μg protein) of total *E. coli* extracts containing the GST protein (control) or GST fused either to the Arabidopsis GAD carboxy-terminal domain (amino acids 473–502) or to the petunia GAD carboxy-terminal domain (amino acids 470–500) were separated on SDS-PAGE, electrotransferred to nitrocellulose membranes, and incubated with ^{35}S-CaM (top). Following autoradiography, the blot was reacted with polyclonal antibodies (Ab.) raised against GST (bottom). The positions of GST and the GST-fusion proteins are indicated by arrows. The lane marked kD contains molecular mass protein markers. B, Alignment of the Arabidopsis (top) and petunia (bottom) GAD-CaMBD sequences. Numbers of the amino-terminal amino acids in each of the domains are indicated on the left. Identical amino acids are boxed.

containing domain. Our results are consistent with the observations that a Leu residue in CaMKII occupies the same hydrophobic cleft in the carboxy-terminal domain of CaM as does the Trp residue of smMLCK (Meador et al., 1993). Thus, CaM/GAD hydrophobic interactions seem to share some features in common with a class of known CaM targets. In addition, we found that electrostatic interactions involving the carboxy-terminal Lys residues also contribute to CaM/GAD binding. Similarly, Lys residues of other CaM targets were found to participate in electrostatic interactions with CaM acidic residues (Clore et al., 1993).

However, the petunia GAD-CaMBD possesses unique features as well. First, atomic resolution analysis revealed a second hydrophobic residue (Leu) in smMLCK and CaMKII that interacts with a hydrophobic pocket in the amino-terminal CaM domain. In these peptides the second hydrophobic residue is separated from the first hydrophobic residue (Trp and Leu) by 12 and 8 residues, respectively. In the GAD-CaMBD there is no conserved Leu residue at the equivalent distances from Trp^{485} (cf. Fig. 11).

Another unique feature of the GAD-CaMBD is the existence of five negatively charged residues. To our knowledge there is no other report of a CaMBD with as many negatively charged residues. Another unique motif, which may serve to counteract the negative charges, are two stretches of positively charged residues flanking the CaMBD on both termini. These motifs are present both in the Arabidopsis and in the petunia GAD-CaMBDs. In other known CaM-target sites, a cluster of positively charged residues is usually found at one of the two termini (Clore et al., 1993). It has been shown by x-ray analysis of Ca^{2+}-CaM/peptide complexes that this positive charge cluster is positioned between two glutamate clusters of Ca^{2+}/CaM, suggesting that through electrostatic interactions with CaM this positive charge cluster is important for orienting CaM binding in the recognition process (Meador et al., 1993).

The Lys residues at the carboxy terminus of the petunia GAD-CaMBD contribute to CaM/GAD interactions in the presence and in the absence of Ca^{2+}. However, because hydrophobic patches of CaM are exposed only in the presence of Ca^{2+}, in the absence of Ca^{2+} hydrophobic CaM/GAD interactions are negligible compared to the apparent electrostatic interactions. Regarding interactions of CaM with target enzymes in the absence of Ca^{2+}, Klee et al. (1986) suggested, based on thermodynamic considerations, that such interactions must exist in vivo for rapid activation of enzymes by Ca^{2+} signals. It should be noted that, whereas we (Table I) and others (Ling et al., 1994) have shown that GAD activity is stimulated by Ca^{2+}/CaM, the mechanism responsible for this stimulation is still unknown. It would be of interest to determine whether rapid activation of GAD in response to stresses (Wallace et al.,

1984) involves CaM/GAD interactions prior to the perception of cytosolic Ca^{2+} signals by CaM.

While studying carboxy-terminal deletions of the petunia GAD-CaMBD, we found that the five amino acids at the very carboxy end are not required for CaM/GAD interactions in vitro. However, because a similar stretch of five amino acids is present at the carboxy end of the Arabidopsis GAD (Fig. 11), these residues are likely to have a function that may or may not be related to the ability of GAD to bind CaM in vivo. In this regard, it has been shown that posttranslational modifications of residues within or adjacent to CaMBDs can affect the ability to bind CaM. For example, negative charges introduced by phosphorylation may block CaM binding to its targets (Hofmann et al., 1994). In each of the petunia and Arabidopsis GAD-CaMBDs, there are four Ser and Thr residues, including one conserved Thr residue (Thr^{496} in the petunia GAD-CaMBD) and one Ser-to-Thr substitution (Thr^{473} in the petunia GAD).

In summary, we performed a fine-mapping analysis of the petunia GAD-CaMBD. Based on the analysis of mutants and of synthetic peptides, we delimited the petunia GAD-CaMBD to residues 470 to 495 (26 amino acids). We demonstrated that hydrophobic and electrostatic interactions are involved in CaM/GAD complex formation. Furthermore, we identified amino acid residues that play a role in these interactions. Our study provides important information for elucidating the functions of the GAD-CaMBD in plants. At the same time, further elucidation of CaM/GAD interactions requires atomic resolution analysis by x-ray and NMR.

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