Identification of Calmodulin Isoform-specific Binding Peptides from a Phage-displayed Random 22-mer Peptide Library*

Received for publication, November 12, 2001, and in revised form, March 15, 2002
Published, JBC Papers in Press, March 18, 2002, DOI 10.1074/jbc.M110803200

Ji Young Choi‡§, Sang Hyoung Lee‡§, Chan Young Park‡§§, Won Do Heo‡†, Jong Cheol Kim‡§, Min Chul Kim‡‡, Woo Sik Chung‡‡, Byeong Cheol Moon‡, Yong Hwa Cheong‡‡, Cha Young Kim‡¶, Jae Hyuk Yoo‡, Ja Choon Koo‡¶, Hyun Mi Ok‡, Seung-Wook Chi¶, Seong-Eon Ryu‡, Sang Yeol Lee‡‡, Chae Oh Lim‡¶, and Moo Je Cho‡†**

From the ‡Division of Applied Life Science (BK21 Program), †Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Chinju 660-701 and the ‡Center for Cellular Switch Protein Structure, Korea Research Institute of Bioscience and Biotechnology, 52 Euh-eun-dong, Yusong, Daejeon 305-333, Korea

Plants express numerous calmodulin (CaM) isoforms that exhibit differential activation or inhibition of CaM-dependent enzymes in vitro; however, their specificities toward target enzyme/protein binding are uncertain. A random peptide library displaying a 22-mer peptide on a bacteriophage surface was constructed to screen peptides that specifically bind to plant CaM isoforms (soybean calmodulin (ScaM)-1 and SCaM-4 were used in this study) in a Ca\(^{2+}\)-dependent manner. The deduced amino acid sequence analyses of the respective 80 phage clones that were independently isolated via affinity panning revealed that ScaM isoforms require distinct amino acid sequences for optimal binding. SCaM-1-binding peptides conform to a 1-5-10 \((FILVW)XXX(FILV)\) motif (where \(X\) denotes any amino acid), whereas SCaM-4-binding peptide sequences conform to a 1-8-14 \((FILVW)XXXXX(FILVW)XXX(FILVW)\) motif. These motifs are classified based on the positions of conserved hydrophobic residues. To examine their binding properties further, two representative peptides from each of the ScaM isoform-biding sequences were synthesized and analyzed via gel mobility shift assays, Trp fluorescent spectra analyses, and phosphodiesterase competitive inhibition experiments. The results of these studies suggest that ScaM isoforms possess different binding sequences for optimal target interaction, which therefore may provide a molecular basis for CaM isoform-specific function in plants. Furthermore, the isolated peptide sequences may serve not only as useful CaM-binding sequence references but also as potential reagents for studying CaM isoform-specific function in vivo.

CaM\(^{2+}\) is a ubiquitous intracellular Ca\(^{2+}\) receptor involved in transducing a variety of extracellular signals (1–3). In contrast to mammals, many plant species belong to a CaM multigene family that encodes various CaM isoforms (4–5). Over 30 genes encoding CaM isoforms are found in the complete nucleotide sequence of Arabidopsis thaliana.\(^2\) CaMs function by modulating or regulating the activities of their target proteins (CaM-binding proteins). More than 30 CaM-binding proteins have been identified, including enzymes such as kinases, phosphatases, and nitric-oxide synthase, as well as receptors, ion channels, G-proteins, and transcription factors (7–9). Plants seem to have evolved a unique repertoire of CaM targets whose homologues in animals do not appear to be modulated by CaMs. The sheer number of CaM isoforms and the diversity of CaM targets imply that these proteins in plants likely modulate a broad spectrum of processes.

CaM is dumbbell-shaped with the N- and C-terminal globular domains separated by a flexible central helix. Each lobe contains two helix-loop-helix Ca\(^{2+}\)-binding motifs referred to as “EF-hands” that are interconnected by a small \(\beta\)-sheet between the two Ca\(^{2+}\)-binding loops (10). Ca\(^{2+}\) binding to CaM induces conformational changes that expose hydrophobic amino acid residues on the surface of both lobes. This creates two hydrophobic pockets that are important for target peptide binding. CaM binds to a large number of proteins through interactions with specific CaM-binding domains. Therefore, one of the most intriguing questions concerning the interaction of CaM with target proteins is: how does a phylogenetically conserved protein like CaM specifically interact with so many different target sites? The determination of the three-dimensional structures of the CaM-peptide complexes greatly aided in answering this question (11–15).

Many known Ca\(^{2+}\)-dependent CaM-binding proteins possess a region that is often characterized by an amphipathic helix consisting of ~20 amino acid residues. This region contains two hydrophobic/aromatic residues that are separated by 12 intervening residues that anchor the peptide to the two lobes of CaM (2). However, sequence analyses based upon this criteria do not always identify the CaM-binding region of a protein, and therefore, the CaM-binding regions of target proteins do not always fit these criteria. Furthermore, the presence of a novel class of CaM-binding proteins that assume a non-helical conformation in the CaM complex has been suggested (16). The CaM-binding domains of these proteins are characterized by the dominance of basic amino acids in contrast to the canonical motif in which hydrophobic amino acids are dominant. An attempt was made to examine the elements common to the many reported CaM-

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* This work was supported by Grant 2000-2-20900-001-1 from KOSEF, the National Research Laboratory (2000), and BK21 Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** These authors contributed equally to this work.

§ These authors contributed equally to this work.

† To whom correspondence should be addressed: Division of Applied Life Science, Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Chinju 660-701, Korea. Tel.: 82-55-751-5957; Fax: 82-55-759-9363; E-mail: mjcho@nongae.gsu.ac.kr.

¶ The abbreviations used are: CaM, calmodulin; BSA, bovine serum albumin; HRP, horseradish peroxidase; gIII, minor coat protein; PDE, 3',5'-cyclic nucleotide phosphodiesterase; ScaM, soybean calmodulin; TBS, Tris-buffered saline; TFE, 2,2,2-trifluoroethanol; GST, glutathione S-transferase; Mops, 4-morpholinepropanesulfonic acid.

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** Munich Information Center for Protein Sequences, mips.gsf.de/proj/thal/.

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However, SCaM-4 has distinguished ability from SCaM-1 in the activation of CaM target enzymes, which can be categorized into three different types (22, 26–29) as follows: 1) enzymes activated equally by both SCaM-1 and SCaM-4 (e.g. phosphodiesterase (PDE), plant Ca$^{2+}$-ATPase, plant glutamate decarboxylase, and CaM-dependent protein kinase II); 2) enzymes activated only by SCaM-1 (e.g. calcineurin, myosin light chain kinase, red cell Ca$^{2+}$-ATPase, and plant NAD kinase); and 3) enzymes activated only by SCaM-4 (e.g. nitric-oxide synthase). SCaM-1 and SCaM-4 also exhibit differences in their Ca$^{2+}$-concentration requirements for target enzyme activation (28). Studies defining CaM-binding sequences and analyzing their interactions with CaM have shed light on the specificity of the interactions between CaM and the particular target molecules.

Here we have adapted an approach for defining the SCaM-1 and SCaM-4-binding peptide sequences using a random peptide bacteriophage display library. Bacteriophage display and affinity selection of phase-displayed peptide libraries, a technique based on screening a library of foreign peptides displayed on the surface of M13 bacteriophage, has proven to be a very useful tool for characterizing a number of protein-protein interactions. Due to the physical linkage of the expressed peptide with its genetic sequence, libraries numbering from $10^8$ to $10^{10}$ peptides have been rapidly screened for a wide variety of applications. This useful tool has been used to map antibody epitopes and to discover peptide ligands for membrane receptors and cytosolic proteins in recent years (30). In this work, we have searched SCaM isoform (SCaM-1 and -4)-favored peptide sequences from a phage display library, and we defined novel SCaM-1- and SCaM-4-specific binding sequences. Furthermore, we have analyzed the interactions between SCaM-1 and SCaM-4 and their respective binding peptides using a variety of biochemical techniques including gel overlay, gel mobility shift, fluorescence spectroscopy, and phosphodiesterase competition assays. Our data show that SCaM isoforms possess different sequences for optimal binding to specific targets. This may provide a molecular basis for CaM isoform-specific function in plants.

**CaM Isoform-specific Binding Peptides**

**Table 1.** Comparison of amino acid sequences of four EF-hands from SCaM-1, SCaM-4, and bovine brain CaM (B-CaM). Consensus shows the most frequently observed amino acid residue in each position of the Ca$^{2+}$-binding loop region of EF hand, which is derived from the comparison of known Ca$^{2+}$-binding proteins (23). Underlined amino acid residues indicate higher than 70% conservation. Ca$^{2+}$-binding ligands are denoted by asterisks, and residues conformed to the consensus are indicated by boldface letters.

| SCaM-1 | DKDGDCITTHE | EF-hand I | SCaM-1 | DADGNTIDFPE | EF-hand I |
| SCaM-4 | DKDGDCITVPE | EF-hand I | SCaM-4 | DADGNTIEPDE | EF-hand II |
| B-CaM  | DKDGDCITTHE | EF-hand I | B-CaM  | DADGNTIDFPE | EF-hand I |
| SCaM-1 | DKDGNGPSAE  | EF-hand II | SCaM-1 | DVGIDQYNEE | EF-hand III |
| SCaM-4 | DKDGNYISAE  | EF-hand III| SCaM-4 | DVGIDQYNEE | EF-hand IV |
| B-CaM  | DKDGNYISAE  | EF-hand III| B-CaM  | DVGIDQYNEE | EF-hand IV |
| **Consensus** | **DKDGNTIDFPE** | **EF-hand I** |

**Fig. 1.** Comparison of amino acid sequences of four EF-hands from SCaM-1, SCaM-4, and bovine brain CaM (B-CaM). Consensus shows the most frequently observed amino acid residue in each position of the Ca$^{2+}$-binding loop region of EF hand, which is derived from the comparison of known Ca$^{2+}$-binding proteins (23). Underlined amino acid residues indicate higher than 70% conservation. Ca$^{2+}$-binding ligands are denoted by asterisks, and residues conformed to the consensus are indicated by boldface letters.

In plant cells, multiple CaM genes code for numerous CaM isoforms in wheat (19), potato (20), Arabidopsis (21), and soybean (22). We have recently cloned five CaM isoforms from soybean (SCaM-1–5). Although some of these isoforms (i.e. SCaM-1–3) are more than 90% identical to mammalian CaM, two (SCaM-4 and SCaM-5) exhibit only a 78% homology with SCaM-1 and are therefore the most divergent isoforms reported thus far in the plant and animal kingdoms. SCaM-4 is considered a bona fide CaM isoform based on the following characteristics. At primary structure level, SCaM-4 has conserved four putative EF-hands and a central linker region, hallmarking structural features of CaM (22). In addition, most of amino acid exchanges occur outside EF-hands, and the number of total amino acid residues are also conserved (148 amino acid residues for SCaM-1 and mammalian CaM, and 149 amino acid residues for SCaM-4 (22)). When compared with the preferred consensus amino acid residues of EF-hands derived from known Ca$^{2+}$-binding proteins (23), the residues in all four Ca$^{2+}$-binding loops of SCaM-4 conform to the consensus (Fig. 1), suggesting that SCaM-4 can bind four Ca$^{2+}$-molecules. All SCaM isoforms including SCaM-4 are ubiquitously expressed in various plant tissues and show similar subcellular localization patterns to the highly conserved SCaM-1 (22, 24). Intriguingly, the cellular level of SCaM-4 rapidly rises upon specific stimuli such as pathogen infection in the same cells constitutively expressing SCaM-1 (25). Furthermore, SCaM-4 has the ability to modulate activity of many CaM-dependent enzymes.
CaM Isoform-specific Binding Peptides

**Fig. 2. Construction of the random peptide library.** Recognition sequences and restriction sites of enzymes are indicated by boxes and **edged lines**, respectively. The **letter N** stands for an equal mixture of A, G, C, and T, and K stands for an equal mixture of Cys and Thr. 

0.1% Tween 20 were preincubated with 0.1% BSA for 1 h at room temperature to remove BSA-binding phages and were bound to the SCaM-coated dishes by incubating them overnight at 4 °C. The plates were washed ten times for 5 min with 1 ml of TBS, 0.1% Tween 20 to remove unbound phages. Phages bound to ScaM-1 or ScaM-4 were eluted in TBS, 0.1% Tween 20 with 2 mg EDTA. The eluted phages were amplified by infecting logarithmic phase E. coli TG1. The helper phage M13K07 was introduced into infected cells to produce phages. The resulting phages were subjected to an additional two rounds of biopanning. SCaM-1 or SCaM-4-binding phages obtained by three rounds of the selection process were plated with 100 μl ampicillin at 37 °C. After clearing the lysate by centrifugation at 15,000 rpm for 10 min at 4 °C, the supernatant (designated as the crude CaM-Pep preparation) was used for the binding assays.

**CaM Gel Overlay Assay**—To prepare horseradish peroxidase (HRP)-conjugated CaMs, we conjugated SCaMs with a maleimide-activated HRP using the EZ-Link maleimide-activated HRP conjugation kit (Pierce) according to the manufacturer’s instructions. Before conjugation, the SCaMs were incubated in 50 mM Hepes, pH 7.0, 0.1 mM dithioretilol at 55 °C for 1.5 h to reduce cysteine residues. The reduced SCaMs were washed and concentrated in degassed phosphate-buffered saline buffer using Centricon C10s (Amicon). After conjugation, the efficiency of SCaMs conjugation to HRP was determined by SDS-PAGE. Conjugation efficiency was usually greater than 90%. Unconjugated residual CaM was removed by gel filtration. CaM gel overlays were performed as described previously (33). Ten μg of GST fusion protein was separated on an 11% SDS-polyacrylamide gel.

**DNA Sequencing**—DNA sequencing was carried out using primers (5′-CTATGATGCAAGGCTGGTATT-3′ and 5′-GGTTAACAAAAAGGAGC-3′) and an Applied Biosystems automatic sequencer (PerkinElmer Life Sciences).

**Peptide Synthesis**—Peptides (Pep A, APAHALFHWGVLGSLIRLV-C18 column, and their amino acid sequences were analyzed.

**Gel Mobility Shift Assays**—The abilities of the synthetic peptides (i.e., Pep A and B) to bind to the SCaM-1 and SCaM-4 isoforms were examined by the relative mobility shifts of the CaM-peptide complexes using 4 M urea polyacrylamide gel electrophoresis in the presence of the peptide and 0.1 mM CaCl2 or 2 mM EGTA. Urea gels contained 13% acrylamide, 4 M urea, 0.375 mM Tris-HCl, pH 8.8, and 0.1 mM CaCl2, or 2 mM EGTA and were run at a constant voltage of 100 V in electrode buffer consisting of 25 mM Tris-HCl, 192 mM glycine, pH 8.3, and 0.1 mM CaCl2, or 2 mM EGTA. The CaM-1 or SCaM-4 isoform and increasing concentrations of Pep A and B (molar ratio: 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, and 2.5) were incubated at room temperature for 1 h in 100 mM Tris-HCl, pH 7.2, 4 M urea, and 0.1 mM CaCl2, or 2 mM EGTA. Glycerol (50%) with tracer bromphenol blue was added before the samples were loaded onto the gel.

**Fluorescence Spectroscopy**—The changes in the microenvironments of the peptide Trp residues upon binding to SCaM were monitored by fluorescence spectroscopy using a PerkinElmer Life Sciences LS50 luminescence spectrometer. The excitation and emission slit widths were 2 nm, and emission spectra were scanned at 10 nm/min with a 1-cm path length cuvette. The Trp residues of Pep A and B were excited in 100 μM buffer (50 mM Mops, pH 7.5, 0.1 mM KCl, 0.1 mM CaCl2) in an EGTA buffer (50 mM Mops, pH 5.0, 0.1 mM KCl, 2 mM EGTA) at room temperature for 30 min were excited at a 295 nm excitation wavelength, and the fluorescence emission spectra in the range 295–560 nm were recorded. An excitation wavelength of 295 nm was used to decrease Tyr fluorescence in ScAM. ScAM was added to the same cuvette from a highly concentrated stock solution to maximize the dilution effects.
**RESULTS**

**Construction and Characterization of the Random 22-mer Peptide Phage Display Library**—The 22-mer peptide display random peptide libraries were constructed using a modified pCANTAB vector, as described in Fig. 2. Random peptides of 22 amino acids in length are expressed at the N terminus of the M13 protein III bacteriophage. Initial transformation of the library produced 4.8 × 10^10 independent clones and subsequent amplification yielded 2.3 × 10^11 pfu/ml. To confirm the diversity of peptide sequences in the library, we randomly picked 80 individual phage clones and determined nucleotide sequences of random peptide regions. In Fig. 3A, the distribution of amino acids in the 22-mer random peptide library is reported as a percentage of the total. All amino acids were uniformly distributed in this library, taking into account the bias inherent in the codon usage ratio. The distribution of amino acid types that appear at each residue position of the sequenced inserts is shown graphically in Fig. 3B. Acidic (Asp and Glu), basic (Lys, Arg, and His), polar (Ser, Thr, Cys, Tyr, Asn, and Gln), and nonpolar (Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, and Met) are relatively proportional to their presence in the genetic code and generate not only linear sequence diversity but also the biochemical diversity desired in a library for screening. ScAM-1 and ScAM-4 Isoforms and the α-series peptides are the α-series and ScAM-4-binding peptides as the β-series. Despite the considerable sequence diversity observed among these clones, their careful alignment yields two distinct binding motifs. Based on the positions of conserved hydrophobic residues, ScAM-1-binding peptides can be fit into a 1-5-10 motif, possessing a consensus sequence of (FILVWXXXFILV) (where X denotes any amino acid). In contrast, ScAM-4-binding peptides conform to a 1-8-14 motif of (FILVWXXXXFAILWVXXXFILVW). In both cases, most of these peptide sequences contain one or more basic amino acid residues, which results in an overall positive net charge for these peptides.

**Circular Dichroism—**CD spectra were obtained on a Jasco J-720 spectropolarimeter using a 1-mm cell at 25 °C. The peptide concentration used for CD measurements was 50 μM, and the solutions included 10 mM Tris-HCl, pH 7.5, and 50% 2,2,2-trifluoroethanol (TFE) (v/v). Secondary structure contents were calculated using the Circular Dichroism Deconvolution program (34).

**Phosphodiesterase (PDE) Competition Assay—**Cyclic nucleotide PDE assays were performed using commercially available bovine heart CaM-deficient PDE (Sigma). The incubation of 100 μM reaction volume contained buffer (100 mM imidazole HCl, 2.56 mM cAMP, 5.13 mM MgSO₄, 1.28 mM CaCl₂) and reaching concentrations of ScAMs (5–200 nM) in the presence (100 nM of either Pep A or Pep B) or absence of peptides. The reaction was started by the addition of PDE (0.3 milliunits/μl). The basal level of enzyme activity was determined in the absence of ScAM, and stimulated activity was determined in the presence of either ScAM-1 or ScAM-4 and CaCl₂. After an incubation at 37 °C for 30 min, the reactions were stopped by placing the reaction tubes in a boiling water bath for 5 min and then on ice for 2 min. Following a brief centrifugation step, 50 μl of alkaline phosphatase (10 units) was added, and the samples were incubated for 10 min at 37 °C. The reactions were stopped by adding 500 μl of 10% trichloroacetic acid. After vortexing, the precipitates were pelleted, and the supernatants (400 μl) were transferred to new tubes. One ml of phosphate reagent (22) was then added, and samples were incubated for 15 min at 37 °C and assayed for Pi content at OD₆₆₀. The dissociation constants (Kₜ) of ScAM-1 and -4 for Pep A and B were calculated from the concentration of ScAM (nM) required to obtain half-maximal PDE activity either in the presence (100 nM) or absence of the peptides. The following equation (35) was used to calculate the dissociation constants: 

\[
K_d = \left( \frac{[P]}{[P] + K - [CaM]} \right) \times \left( \frac{[CaM]}{[CaM] + K} \right)
\]

where \([P]\) represents the total concentration of peptide added, and 

\([CaM]\) and 

K are the concentrations of CaM required to obtain half-maximal activation of PDE in the presence or absence of peptides, respectively. The Kₜ for Pep A or B binding to ScAM-1 or -4 was calculated using the following values: 

\([P] = 100 \text{nM}, K = 9.7 \text{nM} \) (for Pep A) or 10.5 nM (for Pep B) (calculated from the ScAM-1 or -4 activation curves in the absence of Pep A or B), 

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**Fig. 3.** Amino acid distribution in the 22-mer random peptide library. A, a total of 80 peptide sequences from the 22-mer random library was obtained and analyzed as described under “Experimental Procedures.” The percentage of each total amino acid was determined. B, frequency and positional distribution are listed for the amino acid types in the 22-mer random peptide library. The amino acids were divided into acidic (Asp and Glu), basic (Lys, Arg, and His), nonpolar (Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, and Met), and polar (Ser, Thr, Tyr, Asn, and Gln) species. The frequency of each amino acid type at each residue position is indicated as follows: acidic, dotted bars; basic, open bars; nonpolar, hatched bars; and polar, filled bars.

**ScAM-4 via an enzyme-linked immunosorbent assay-based assay (see “Experimental Procedures”).** Approximately 90% of the isolates exhibited strong ScAM-1- or -4-binding activity (data not shown). Each of the 80 independent phages reacting strongly in the enzyme-linked immunosorbent assay were selected from the ScAM-1- and ScAM-4 biopanning, and their nucleotide sequences were determined. In Fig. 4, their deduced amino acid sequences are shown in random peptide regions, which are aligned to reveal consensus sequences. For convenience, we designated ScAM-1-binding peptide sequences as the α-series and ScAM-4-binding peptides as the β-series. Despite the considerable sequence diversity observed among these clones, their careful alignment yields two distinct binding motifs. Based on the positions of conserved hydrophobic residues, ScAM-1-binding peptides can be fit into a 1-5-10 motif, possessing a consensus sequence of (FILVWXXXFILV) (where X denotes any amino acid). In contrast, ScAM-4-binding peptides conform to a 1-8-14 motif of (FILVWXXXXFAILWVXXXFILVW). In both cases, most of these peptide sequences contain one or more basic amino acid residues, which results in an overall positive net charge for these peptides.
CaM Isoform-specific Binding Peptides

Identification of SCaM-1- or -4-specific Peptides by Gel Overlay Assay—To verify the specificity of SCaM-binding phase isolation using biopanning, a CaM gel overlay assay was performed (24). First of all, three of the most frequently isolated peptides obtained from each of the SCaM-binding phases (i.e. α1, α2, and α3 for SCaM-1 and β1, β2, and β3 for SCaM-4) were expressed as GST fusion proteins using the vector pGEX-KG (30). As shown in Fig. 5, these GST fusion proteins exhibited molecular masses of ~29 kDa by SDS-PAGE, consistent with the predicted values of ~3 kDa for the SCaM peptide plus ~26 kDa for the GST protein. In the gel overlay assays, all α peptide fusion proteins exhibited positive signals with SCaM-1-HRP (Fig. 5A), whereas SCaM-4-HRP resulted in no signal. Conversely, for the β peptide fusion proteins, only SCaM-4-HRP exhibited positive binding. Interestingly, CaM binding signals for these fusion proteins correlated with their frequency of isolation in the biopanning. For example, α1 and β1, the most frequently isolated sequences for SCaM-1 and SCaM-4 binding, respectively, produced the strongest signals among the tested α and β peptides in the gel overlays (Fig. 5, A and B). In either case, the negative control (i.e. GST protein alone) exhibited no binding to either of the SCaM-HRP probes. In addition, no significant signal was detected when the gel overlay assays were performed in the presence of an EGTA-containing buffer (data not shown). Thus, these results indicate that these peptide sequences specifically bind to the corresponding SCaM-1 or SCaM-4 in a Ca2+-dependent manner.

Gel Mobility Shift Assays—The gel overlay assays confirmed the binding of SCaM to the isolated peptide sequences. However, they did not provide quantitative information for these interactions. To examine further the SCaM-binding specificity of the isolated peptide sequences, the most frequently isolated peptide sequence was selected from each SCaM-1 or SCaM-4-binding peptide sequence. These are designated as Pep A (APAHLFHVGLGLIRLVLFLS) and Pep B (CNRLRSLRNYGGYVLVLS), respectively. The synthesized peptides were then tested by gel mobility shift to assess their interactions with the SCaM isoforms. Complexes formed between the SCaM isoforms and either Pep A or Pep B were confirmed by gel electrophoresis in the presence of 4 M urea. Urea was used since it dissociates low affinity and nonspecific complexes, leaving only the higher affinity and higher specificity complexes behind. As the ratio of peptide to Ca2+-SCaM increased, we observed a band shift due to the formation of a complex between the peptide and Ca2+-SCaM (Fig. 6, A and D). In the absence of peptide, SCaM migrates as a single band (data not shown). When the ratio of peptide to SCaM was equal, a mo-
or Pep B was determined in the presence of either 0.1 mM CaCl2 or 2 mM EGTA. Purified SCaM-1 or SCaM-4 was incubated with increasing amounts of Pep A or B (Pep A or B versus SCaM-1 or -4 molar ratios are indicated), and then samples were separated by nondenaturing PAGE in the presence of 4 μl urea. The gels were stained with Coomassie Brilliant Blue. A indicates SCaM-1 + Pep A + Ca2+; B indicates SCaM-1 + Pep A + EGTA; C indicates SCaM-4 + Pep A + Ca2+; D indicates SCaM-4 + Pep B + Ca2+; E indicates SCaM-4 + Pep B + EGTA; and F indicates SCaM-1 + Pep B + Ca2+. The arrows in A and D indicate the SCaM-1-Pep A or SCaM-4-Pep B complexes, respectively. Free peptide is not apparent because it had run out of the gels by the time electrophoresis was stopped.

Fig. 7. Trp fluorescence spectra of peptide binding to SCaM isoforms. R, fluorescence spectra were measured in the presence of Ca2+-EGTA using a PerkinElmer Life Sciences LS50 luminescence spectrometer. An excitation wavelength of 295 nm was used to reduce Trp fluorescence from SCaM-1 (A) and SCaM-4 (B). SCaM-1 or -4 (2.5 μM) was added to a solution containing 1.9 μM Pep A or B in the presence of either 0.1 mM Ca2+ or 2 mM EGTA.

Circular Dichroism Spectra—To examine the molecular basis of the binding specificity for the plant CaM isoforms, we characterized the secondary structures of the model peptides. Because shorter peptides tend to exist as flexible coils in pure water, we determined the secondary structures of the model peptides in the presence of 50% TFE, a compound that stabilizes secondary structures (38). In Fig. 8, the CD spectra for Pep A and Pep B obtained at 25 °C are presented. As can be seen, the spectra are very similar to each other. Both contain a minimum at 206–208 nm with a shoulder at around 222 nm, indicating the dominant presence of α-helices. From the CD spectra, the α-helix contents of Pep A and Pep B are estimated to be 72 and 76%, respectively. Thus, both Pep A and Pep B are likely to bind CaM isoforms as helices. In addition, in their SCaM-1-bound forms, both peptides showed increases in their α-helical content, further supporting this theory (data not shown).

Peptide-dependent Inhibition of CaM-activated PDE Activity—Finally, the relative affinities of Pep A and Pep B for the SCaM isoforms were determined by using a competition assay for PDE, an enzyme that is activated equally by both SCaM-1 and SCaM-4.
CaM Isoform-specific Binding Peptides

Fig. 8. CD spectra of Pep A and Pep B. CD spectra of Pep A (—) and Pep B (—) were measured in 50% TFE (v/v). Five consecutive scans (250–200 nm) for each sample were averaged to generate a spectrum.

Fig. 9. Effect of Pep A and Pep B on the activation of PDE by SCaM isoforms. The dose-dependent inhibitions of PDE activity by Pep A and Pep B are shown. The inhibitory activities were measured in the presence of Ca²⁺ and a fixed concentration (120 nM) of SCaM-1 (A) or SCaM-4 (B) with increasing concentrations of Pep A or Pep B. PDE activity was measured in the presence of varying concentrations of SCaM-1 or SCaM-4 and either in the presence or absence of fixed concentrations (100 nM) of Pep A (C) or Pep B (D). Results are shown as the means obtained from three independent assays (n = 3).

and SCaM-4 (22). In Fig. 9, A and B, the effect of increasing peptide concentrations on PDE activity is shown for a fixed concentration (120 nM) of SCaM-1 or SCaM-4, a concentration sufficient for the maximal activation of PDE. A gradual decrease in PDE activity was observed with increasing concentrations of the relevant peptides. Half-maximal inhibition of PDE activation by SCaM-1 and SCaM-4 was obtained at ~120 nM Pep A and ~80 nM Pep B, respectively. Up to a concentration of 600 nM, Pep A had no effect on the activation of PDE by SCaM-4 and a concentration of more than 1 μM was required to reach half-maximal inhibition. Pep B, on the other hand, was a slightly more potent inhibitor of PDE activation by SCaM-1, inhibiting at concentrations above 400 nM, and although similar to Pep A, half-maximal inhibition was not reached at concentrations below 1 μM. To determine $K_i$ values for these peptides in the activation of PDE by SCaMs, the CaM dose-dependent activation of PDE was determined in the presence (100 nM) or absence of the peptides (Fig. 9, C and D). The activation curves shifted to the right in the presence of the peptides, indicating a competition occurring between PDE and the peptides for the SCaMs. The $K_i$ values for Pep A and Pep B for their inhibition of the activation of PDE by SCaM-1 or SCaM-4 was determined to be 31.0 and 28.5 nM, respectively (Fig. 9, C and D). These experiments suggest that Pep A and Pep B have at least a 10-fold specificity for SCaM-1 and SCaM-4, respectively.

DISCUSSION

We have shown previously (22, 26–28, 33) that SCaM isoforms exhibit differences in their abilities to activate target enzymes. Importantly, some CaM isoforms competitively inhibit the activation of certain enzymes by other CaM isoforms, exhibiting a reciprocal regulation of target enzymes (26, 28). In these in vitro enzyme assays, the SCaM isoforms often possessed significantly different affinities for a given enzyme. For example, with the activation of plant Ca²⁺-ATPase, SCaM-1 has ~20-fold higher affinity than SCaM-4 (28). Furthermore, in gel overlay assays of various plant CaM-binding proteins, these isoforms exhibited different binding intensities, suggesting that different CaM isoforms might require distinct primary sequences for optimal target binding (24). In this investigation, in which we took advantage of a phage-displayed random peptide library, we identify the peptide sequences that interact with specific SCaM isoforms. Surprisingly, two distinct families of peptides that specifically bind to SCaM-1 or SCaM-4 isoforms in a Ca²⁺-dependent manner were identified in this study. These peptides fit into a 1-5-10 $(\text{FILVW})$ motif for the SCaM-1-binding peptides and a 1-8-14 $(\text{FILVWXXXXXX(FILVW)})$ motif for the SCaM-4-binding peptides. Rhoads and Friedberg (17) originally identified these motifs as Ca²⁺-dependent brain CaM-binding sequences, based upon the positions of conserved hydrophobic amino acid residues. These hydrophobic residues play an important role in anchoring peptides to target binding pockets of CaM via a hydrophobic interaction. It is also noteworthy that these Ca²⁺-dependent CaM-binding motifs require basic residues, which are important in stabilizing the binding interaction by forming salt bridges. Consistent with this, all of the isolated peptides in this study were determined to be basic.

It is quite remarkable that plant CaM isoforms exhibit differing optimal sequence preferences for their target interactions in vitro. This is most likely due to structural differences between CaM isoforms. For example, SCaM-1 and SCaM-4 are ~22% different in regard to their primary amino acid sequences. What is the significance of this finding in terms of the function of CaM in plant cells? One intriguing possibility is that certain CaM isoforms may require specific binding targets thereby leading to unique cellular responses. This is consistent with the idea that these CaM isoforms evolved independently following segregation from a progenitor CaM, as predicted from phylogenetic analyses of these CaM isoforms (22). However, we should also point out that these isolated peptides could be the optimal binding sequences for given CaM isoforms, which are not found in nature. Indeed, in CaM gel overlay assays using plant cell extracts, we could hardly find binding proteins specific for the CaM isoforms (24). In addition, so far none of the enzymes tested in vitro in our laboratory exhibit specific binding to particular CaM isoforms. Furthermore, the differences in binding affinity between Pep A and Pep B to the SCaM isoforms were no greater than 10-fold. Therefore, it is reasonable to speculate that a large number of CaM-binding proteins bind to both isoforms, given a certain degree of affinity differences, and therefore only a few CaM isoform-specific binding proteins may exist in plants.

We reported previously that SCaM isoforms are different in their abilities to activate various target enzymes (28, 33). We
examined whether there is a correlation between the differential target binding preferences for the activation profiles of SCaMs. Interestingly, in the case of α-CaMKI, which has a 1-5-10 CaM-binding motif, the 1-5-10-favoring SCaM-1 activated this enzyme and at a much lower concentration than SCaM-4 (~10-fold less, K_m = 22 versus 275 nM). Conversely, SCaM-4 was >4-fold better than SCaM-1 in the activation of nitric-oxide synthase, a 1-8-14-type enzyme (28). These positive correlations further support the biological relevance of our findings.

Previously, two other groups (39, 40) reported isolating CaM-binding sequences using similar strategies. Dedman et al. (39) pioneered this type of work using a random 15-mer library, shorter than the typical CaM-binding domains, to screen for CaM-binding peptides. Among the 28 isolated peptides, Trp was always present and often (11 peptides) present in the first variable position of the random peptide inserts. Additionally, 17 of the peptides contained Trp-Pro sequences. Nevalainen et al. (40) used a random 8-mer peptide library and similarly found the Trp residue and the Trp-Pro combination in the variable position of the random peptide inserts. Additionally, 17 of the peptides contained Trp-Pro sequences. Nevalainen et al. (40) used a random 8-mer peptide library and similarly found the Trp residue and the Trp-Pro combination in the variable position of the random peptide inserts. Additionally, 17 of the peptides contained Trp-Pro sequences. Nevalainen et al. 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**CaM Isoform-specific Binding Peptides**
Identification of Calmodulin Isoform-specific Binding Peptides from a Phage-displayed Random 22-mer Peptide Library

Ji Young Choi, Sang Hyoung Lee, Chan Young Park, Won Do Heo, Jong Cheol Kim, Min Chul Kim, Woo Sik Chung, Byeong Cheol Moon, Yong Hwa Cheong, Cha Young Kim, Jae Hyuk Yoo, Ja Choon Ko, Hyun Mi Ok, Seung-Wook Chi, Seong-Eon Ryu, Sang Yeol Lee, Chae Oh Lim and Moo Je Cho

J. Biol. Chem. 2002, 277:21630-21638. doi: 10.1074/jbc.M110803200 originally published online March 18, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M110803200

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