Characterization of novel calmodulin-binding peptides with distinct inhibitory effects on calmodulin-dependent enzymes

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We describe the isolation and interaction with calmodulin (CaM) of two 10-amino-acid peptides (termed peptides 1 and 2; AWDTVRISFG and AWPSLQAIRG respectively) derived from a phage random peptide display library. Both peptides are shorter than previously described CaM-binding peptides and lack certain features found in the sequences of CaM-binding domains present in CaM-activated enzymes. However, ^{1}H NMR spectroscopy and fluorimetry indicate that both peptides interact with CaM in the presence of Ca^{2+}. The two peptides differentially inhibited CaM-dependent kinases I and II (CaM kinases I and II) but did not affect CaM-dependent phosphodiesterase. Peptide 1 inhibited CaM kinase I but not CaM kinase II, whereas peptide 2 inhibited CaM kinase II, but only partially inhibited CaM kinase I at a more than 10-fold higher concentration. Peptide 1 also inhibited a plant calcium-dependent protein kinase, whereas peptide 2 did not. The ability of peptides 1 and 2 to differentially inhibit CaM-dependent kinases and CaM-dependent phosphodiesterase suggests that they may bind to distinct regions of CaM that are specifically responsible for activation of different CaM-dependent enzymes.

INTRODUCTION

Calmodulin (CaM), a ubiquitous cytoplasmic protein found in all eukaryotic cells, plays a central role as a Ca^{2+}-sensitive target in signal transduction. Ca^{2+}/CaM interacts with a variety of target molecules, many of them enzymes, to mediate responses to changes in the cytoplasmic Ca^{2+} concentration. Thus CaM has been found to be involved in a wide variety of essential functions, ranging from cell cycle progression to neurotransmission [1–4].

In the non-target-bound state, CaM has a 'dumbbell' shape, with the N-terminal and C-terminal domains separated by a flexible central helix. The C-terminal domain contains two high-affinity Ca^{2+}-binding sites ($K_d \sim 10^{-7}$ M), while the N-terminal lobe contains two lower-affinity sites ($K_d \sim 10^{-5}$ M) [5]. Binding of Ca^{2+} to CaM induces conformational changes that expose hydrophobic amino acid residues on the surface of both lobes, creating two hydrophobic pockets. CaM binds to a large number of target proteins through interactions with specific CaM-binding domains. Although the primary sequences of these various domains show little primary structural identity, they contain a consistent feature of two aromatic or long-chain hydrophobic amino acids separated by 8–12 residues [6–10]. Upon binding to a target domain the central helix of CaM adopts a loop structure to allow the lobes to make multiple contacts with the target and to accommodate the bulky hydrophobic residues [7,8]. Thus the ability of CaM to accommodate different target sequences in its hydrophobic pockets is believed to be due to flexibility of the central helix and local rearrangement of side chains in the binding surface.

Studies involving NMR, X-ray crystallography, chemical modification of specific regions of CaM, site-directed mutagenesis of CaM, preparation of CaM–troponin C chimeras and analysis of CaM fragments or, alternatively, site-directed mutagenesis of CaM-binding domains in target proteins, have shed light on the specificity of interactions between CaM and particular target molecules [7–9,11–25]. Yet, given the variability in primary amino acid sequences of CaM-binding proteins, many questions remain concerning the detailed mechanism that allows CaM to recognize and discriminate between more than 20 target molecules. Resolution of this issue is likely to depend on a detailed characterization of specific CaM–target complexes, as well as on defining novel CaM-binding sequences and analysing their interactions with CaM, particularly in the context of the intact target protein.

A number of studies of competition between various CaM-binding peptides for CaM have been carried out. For example, the 25-amino-acid CaM-dependent kinase II (CaM kinase II)-binding peptide has been shown to inhibit both CaM kinase II and another target enzyme, CaM-dependent phosphodiesterase (CaM PDE) [26]. Furthermore, melittin and mastoparan share features of CaM target-binding domains and compete with CaM-activated enzymes for binding to CaM [27,28]. Here we identify 10-amino-acid CaM-binding peptides, synthesized based on sequences isolated from a phage random peptide display library. The peptides display specificity with respect to inhibition of CaM-dependent enzyme activity and may thus contribute to the further understanding of CaM–target protein interactions.

EXPERIMENTAL

Materials

Neurospora and bovine testes CaMs, AGI-X2 anion-exchange resin, histone III-S and jellyfish aequorin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CaM PDE was from Boehringer Mannheim. Factor Xa was from New England

Abbreviations used: CaM, calmodulin; CaM kinase I, CaM-dependent kinase I; CaM kinase II, CaM-dependent kinase II; CaM PDE, CaM-dependent phosphodiesterase; atCDPK6, *Arabidopsis thaliana* calcium-dependent protein kinase; gpIII, minor coat protein; pAq, aequorin-binding peptide; peptides 1 and 2, CaM-binding peptides of sequences AWDTVRISFG and AWPSLQAIRG respectively.

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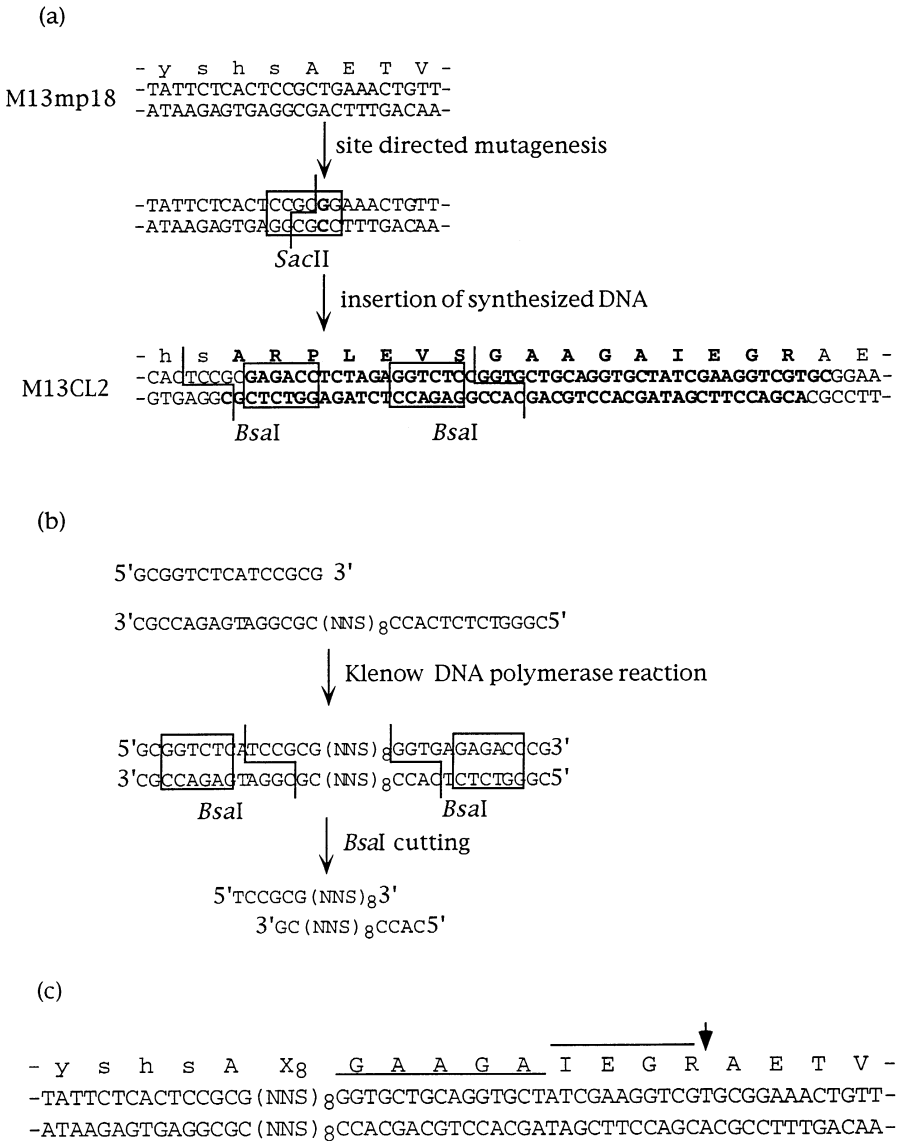

Figure 1 Construction of the random octapeptide library

Amino acid sequences in the signal peptide and the mature protein are indicated in lower and upper case respectively. 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 S stands for an equal mixture of G and C. (**a**) Procedure for construction of the peptide library vector M13CL2. The amino acid sequence around the N-terminus of gpIII and the corresponding DNA sequence are shown. Bold letters correspond to the substituted or inserted residues. (**b**) Procedure for construction of the double-stranded DNA coding for random peptides. (**c**) Amino acid sequence around the random peptide, indicated as X_8, and the corresponding DNA sequence. The flexible-arm sequence is underlined. The recognition sequence for Factor Xa is indicated by a overline, with an arrowhead at the site of proteolysis.

BioLabs. *N*-Hydroxysuccinimide long-chain (NHS-LC)-biotin and streptavidin were from Pierce. Recombinant CaM kinase I was prepared as described in [29], and CaM kinase II was prepared as described in [30]. *Arabidopsis thaliana* calcium-dependent protein kinase (atCDPK6) was prepared as a bacterial His-tagged fusion protein (H. Yan, unpublished work).

Construction of a phage display library

A phage library was produced based on the concept described by Scott and Smith [31], in which random peptide sequences were expressed on the N-terminus of the minor coat protein (gpIII) of filamentous phage. The phage vector M13mp18 [32] was modified to make a vector for the library (Figure 1a). The DNA fragment carrying the chloramphenicol acetyltransferase gene and its promoter was isolated from the plasmid vector pHSG396 [33] using PCR and placed in the *lac* promoter region of M13mp18 by replacing the *Kas*I–*Sal*I fragment. A single-base substitution resulting in a *Sac*II site was made in the N-terminal region of gene III using site-directed mutagenesis [34]. The synthesized DNA was inserted at the *Sac*II site to introduce two *Bsa*I sites and a sequence coding for junction amino acid residues. The resulting phage peptide library vector was named M13CL2.

Double-stranded DNA coding for random peptides was produced as shown in Figure 1(b). Single-stranded DNA encoding eight-residue random peptides and two *Bsa*I sites was synthesized and converted into double-stranded DNA using Klenow DNA polymerase and an appropriate primer. The DNA was cut by

*Bsa*I to produce cohesive ends on both sides and substituted for the small *Bsa*I fragment of M13CL2. The resulting DNA sequence and amino acid sequence of the N-terminal region of the gpIII are shown in Figure 1(c). In the fusion protein, an eight-residue random peptide is connected to the N-terminus of gpIII by a flexible arm and a recognition sequence of the protease Factor Xa. Twelve electroporations, each using 0.5 μg of the DNA constructed above and 400 μl of electrocompetent *Escherichia coli* DH1 cells, yielded 1.8×10^8 transformants producing infectious phage. Multiple cycles of phage infection, which would cause a bias in random peptide sequences, were avoided by using F^- cells for transformation and phage production. After 2 h of non-selective outgrowth in 2.4 litres of 2 × YT broth, the cells were grown for 12 h under chloramphenicol selection (10 μg/ml), and the phage were isolated as described [35].

Screening of the phage random peptide library

The screening cycle of a phage library consists of two steps, phage selection for affinity and phage amplification. Although the selection should happen only in the first step, the second step may also non-specifically select phage with good infectivity. To avoid any undesirable non-specific selection, the phage library in this study was designed so that Factor Xa treatment would result in all the phage having the same gpIII N-terminus as that of M13mp18. Phage were then treated with Factor Xa before infection in the following experiments. The library was screened with biotinylated spinach CaM or biotinylated jellyfish aequorin (10 μg) fixed on a streptavidin-coated dish (35 × 10 mm polystyrene Falcon dishes). Approx. 1×10^{12} plaque-forming units (p.f.u.) of phage library in TBS (150 mM NaCl in 50 mM Tris/HCl, pH 7.5) with 100 μM biotin and 0.5 % Tween-20 was added in the absence of EGTA or EDTA. After binding overnight at 4 °C, the plates were washed six times for 1 min with 1 ml of TBS/Tween, incubated on a rocker for 2 h at 37 °C with TBS/Tween containing 100 μM biotin, and washed again six times for 1 min with 1 ml of TBS/Tween. The remaining phage were then eluted by incubation on a rocker for 2 h at 37 °C with 1 or 10 μM $CaCl_2$ in TBS/Tween with 100 μM biotin. The eluted phage were concentrated in Centricon-100 spin columns to approx. 50 μM and treated with 5 μl of Factor Xa protease overnight at 4 °C to digest the random peptide from gpIII before amplification in *E. coli* [31]. To further enrich tightly binding phage, another round of panning was carried out by plating 1×10^{12} p.f.u. per plate of the amplified phage population. The enrichment was monitored by titrating the number of eluted phage after each round of panning. Two rounds of screening with CaM resulted in an increase in the ratio of eluted to input p.f.u. by about 10^4-fold. Sequencing of clones derived from the second round of panning revealed that the sequences WDTVRISF (termed clone 1) and WPSLQAIR (termed clone 2) represented 60 % and 20 % respectively of the clones when a total of 100 clones were sequenced. The remaining clones appeared 1–3 times each (see Figure 2). Three rounds of affinity selection with CaM gave a virtually uniform population of phage clones identical with clone 1.

Certain features of the amino acid sequences obtained in the present study were similar to those of CaM-binding peptides identified by similar methods in a previous study [36]. Notably, in the previous study, EGTA was used to elute binding phage, whereas in the present study 0.5 % Tween in the presence of 1–10 μM Ca^{2+} was used in the elution buffer. Therefore the elution characteristics of clones 1 and 2 were studied further. Both clones were eluted in 10^2–10^3-fold greater numbers in the presence of detergent (0.5 % Tween) than in its absence. The presence of Ca^{2+} (100 μM) or EGTA (2 mM) in the elution step had little effect on the number of eluted phage (results not shown). In the presence of detergent the phage detached from CaM gradually and more phage could be eluted at each successive detergent wash. Thus, in the panning for CaM binding, the repeated detergent washes presumably enriched phage that bound to the hydrophobic sites of CaM and were the most resistant to detergent washes.

DNA sequencing

After two rounds of panning, samples of eluted phage were sequenced (USB Sequenase Version 2.0) using primers near the random peptide cloning site.

Peptide synthesis

Peptides were synthesized at The Rockefeller University Protein Sequencing Facility with an Applied Biosystems Model 430 instrument by fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry, utilizing 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate activation coupled with *N*-methylpyrrolidone. Peptides were desalted and purified by reverse-phase HPLC using a C_{18} column, and analysed by amino acid analysis and MS.

Determination of peptide concentrations

The concentrations of peptide 1, peptide 2 (for sequences, see below) and the aequorin-binding peptide (pAq), as well as the tryptic fragments F12 and F34 of CaM, were determined by amino acid analysis at The Rockefeller University Peptide Sequence Facility. CaM concentrations were determined either by weighing lyophilized powder or by Bradford protein assay. The purity of CaM preparations and of fragments F12 and F34 was confirmed by SDS/PAGE.

Enzyme assays

CaM PDE was assayed in 100 μl of a reaction mixture containing 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.3 mg/ml BSA, 10 μM $CaCl_2$, 3 μM CaM, 10 μM cAMP and [^{3}H]cAMP. The reaction was stopped after incubation for 10 min at 30 °C by boiling the samples for 1 min. *Crotalus atox* snake venom (100 μg/ml) was added and the samples were incubated for an additional 10 min at 30 °C. The second reaction was stopped by adding 1 ml of AGI-X2 anion-exchange resin in a slurry, mixing well and spinning in a microcentrifuge at 10000 ***g*** for 5 min. Samples of 200 μl of clear supernatant from each sample were analysed by liquid scintillation spectrometry.

Assays of CaM kinases I and II were carried out essentially as described in [29]. The reaction mixture contained 50 mM Hepes (pH 7.5), 10 mM magnesium acetate, 50 μM EGTA, 5 mM dithiothreitol, 1 μM CaM, 200 μM $CaCl_2$ (if present), 200 μM peptide substrate and 5–50 μM [γ-^{32}P]ATP [specific radioactivity $(2–5) \times 10^2$ c.p.m./pmol]. The samples were brought to 30 °C and the reaction was started by addition of CaM kinase I or II. After 10 min the reaction was stopped by adding acetic acid to a final concentration of 10 %, and an aliquot of each sample was spotted on to P81 filter paper (Whatman). The papers were washed with deionized water and ^{32}P incorporation into peptide was measured by liquid scintillation spectrometry (Çerenkov counting).

atCDPK6 was assayed in a reaction mixture of 25 μl containing 1 μg of purified protein in 20 mM Tris/HCl, pH 7.4, 6 mM

$MgCl_2$, 1 mM EGTA (if present), 1 mM $CaCl_2$ (if present) and 25 μg of histone III-S as substrate. Reactions were initiated by the addition of 5 μCi of [γ-^{32}P]ATP in a final ATP concentration of 50 μM and incubated for 20 min at room temperature. Reaction mixtures were spotted on to Whatman 3MM filter paper, followed by immersion in ice-cold 10 % (w/v) trichloroacetic acid containing 10 mM disodium pyrophosphate. Filters were washed for 3 × 15 min with the above solution and then for 15 min in 95 % ethanol. Dried filters were counted for radioactivity in a scintillation counter.

Spectrofluorimetry

Tryptophan fluorescence spectra were measured as described [37] in Ca^{2+} buffer (50 mM Mops, pH 7.5, 0.1 M KCl, 0.1 mM $CaCl_2$) or in EGTA buffer (50 mM Mops, pH 7.5, 0.1 M KCl, 2 mM EGTA) using a Perkin–Elmer LS50 Luminescence Spectrometer. An excitation wavelength of 295 nm was used to decrease Tyr fluorescence from CaM.

Preparation of N- and C-terminal tryptic peptides of CaM

The N- and C-terminal tryptic fragments (amino acid residues 1–75 and 78–148 respectively) of scallop testes CaM were prepared and purified as described [38].

NMR spectroscopy

One-dimensional ^{1}H NMR spectra of recombinant *Xenopus laevis* CaM (0.5 mM) as a function of peptide concentration (0–0.25 mM) both in the absence and in the presence of 2.5 mM $CaCl_2$ were recorded at 25 °C on a UNITY-Plus 500 spectrometer. The spectral width was 6000 Hz with digital resolution of 0.75 Hz. All spectra were recorded with 128 transients.

A sample of Ca^{2+}-free CaM for ^{1}H NMR analysis of CaM–peptide complexes was prepared by dissolving an aliquot of *Xenopus* CaM in 50 mM EGTA. The EGTA was then removed by desalting the sample through a PD-10 column (Pharmacia), which was made Ca^{2+}-free prior to use by washing with 5 ml of 0.1 M EGTA and 25 ml of Ca^{2+}-free 50 mM NH_4HCO_3, pH 8.0 (decalcified by elution through Bio-Rad Chelex resin). ^{1}H NMR spectra of the CaM–peptide complexes in the presence of Ca^{2+} were recorded after direct addition of an appropriate aliquot of $CaCl_2$ solution to the CaM–peptide solution in the NMR tube.

RESULTS

Isolation of CaM-binding peptides

Peptide sequences derived from the random peptide library screened with CaM displayed a number of conserved features (Figure 2). With only a few exceptions, the first variable amino acid in all sequences was Trp. Most of the sequences could be grouped into two types; the final variable residue in all 'type 1' sequences was Phe, whereas all 'type 2' sequences had Pro immediately following the initial Trp. The most frequent sequence, WDTVRISF (termed peptide 1), was a type 1 sequence, and it alone represented 60 % of all sequences obtained. Ser or Thr frequently appeared in the third, and Val in the fourth, position in type 1 sequences. The second most frequent sequence, WPSLQAIR (termed peptide 2), alone represented 20 % of all sequences obtained and was a type 2 sequence. Leu appeared frequently in the fourth position in type 2 sequences.

Jellyfish aequorin, which is also a Ca^{2+}-binding protein, was used in an independent affinity screening of the phage library. No peptides similar to the CaM-binding peptides were obtained (Figure 2). The most frequent aequorin-binding peptide, HPWGYPKI (pAq), was used as a control peptide in subsequent enzyme assays that analysed CaM-binding peptides 1 and 2.

A

Type 1 peptides	Type 2 peptides
AWDTVRISFG*	AWPSLQAIRG*
AWTPSASRFG	AWPTLSKIAG
AWESVRTHFG	AWPRPCRHSG
AWSPSASRFG	AWPPPARVIG
AWASVRTHFG	AWPQLQRLIG
AWDTVRICFG	AWPPLSSVLG
AWESCGTHFG	

B

AHPWGYPKIG**
AHRGGTPKIG
AHPWTRTISG
AHPWTRTDSG
AWNITWSFSG
AFQNMRMAGG
APAKPRAATG
AFHNQRMAGG
AGTHYHASPG

Figure 2 Peptide sequences derived from the phage display library by affinity selection with CaM (A) or pAq (B)

The first amino acid, Ala (A), and the final amino acid, Gly (G), are present in the gpIII coat protein. The asterisks indicate the representative peptide in each group that was synthesized and used for further analysis.

Peptides 1 and 2 bind to CaM in a Ca^{2+}-dependent fashion

The peptides that were most frequently obtained in each group were synthesized based on the cloned octapeptide sequence expressed by clones 1 (WDTVRISF) and 2 (WPSLQAIR), and the aequorin-binding clone (HPWGYPKF). Ala and Gly were included at the N- and the C-terminus respectively, since these two amino acids flanked the random sequence in the phage gpIII coat protein. Peptide 2 and pAq were dissolved in water, whereas, due to its poor solubility in water, peptide 1 was dissolved in 10 % DMSO as a 500 μM stock solution.

The interactions of peptides 1 and 2 with CaM were examined in the absence and the presence of Ca^{2+} using ^{1}H NMR analysis. In the absence of Ca^{2+}, the ^{1}H NMR spectra of CaM plus either peptide were similar to that of Ca^{2+}-free CaM [39], indicating that peptides 1 and 2 did not induce major conformational changes in CaM. In contrast, in the presence of Ca^{2+}, the NMR spectra of Ca^{2+}-bound CaM were significantly perturbed by the addition of peptide 1 or 2. Notably, high-field shifted aromatic resonances were affected by the addition of either peptide, suggesting conformational changes in the hydrophobic regions of CaM (Figure 3). However, the interaction of either peptide with Ca^{2+}/CaM was weaker than that previously observed with the myosin light chain kinase peptide M13 [7].

The interaction of peptide 2 with CaM was also examined by taking advantage of the intrinsic fluorescence of the Trp residue in the peptide sequence. Excitation and emission wavelengths were 295 nm and 300–500 nm respectively (Figure 4). The fluorescence intensity increased and was blue-shifted when 2.7 μM CaM was added to 1.8 μM peptide 2 in the presence of 0.1 mM Ca^{2+} (Figure 4A). No change was observed when CaM was added in the presence of 2 mM EGTA (Figure 4B). The blue shift results from movement of Trp into a more hydrophobic environment, suggesting the occurrence of a Ca^{2+}-dependent interaction of peptide 2 with hydrophobic regions of CaM.

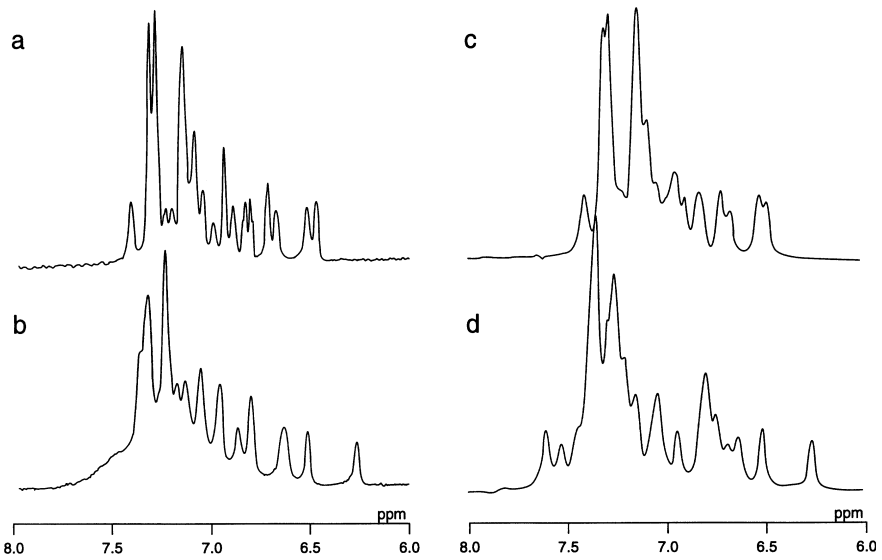

Figure 3 Effects of peptides 1 and 2 on the ^{1}H NMR spectrum of CaM

The ^{1}H NMR spectrum of the aromatic region of CaM is shown in the presence of either peptide 1 with and without 10 mM $CaCl_2$ (**a** and **b** respectively) or peptide 2 with and without 10 mM $CaCl_2$ (**c** and **d** respectively).

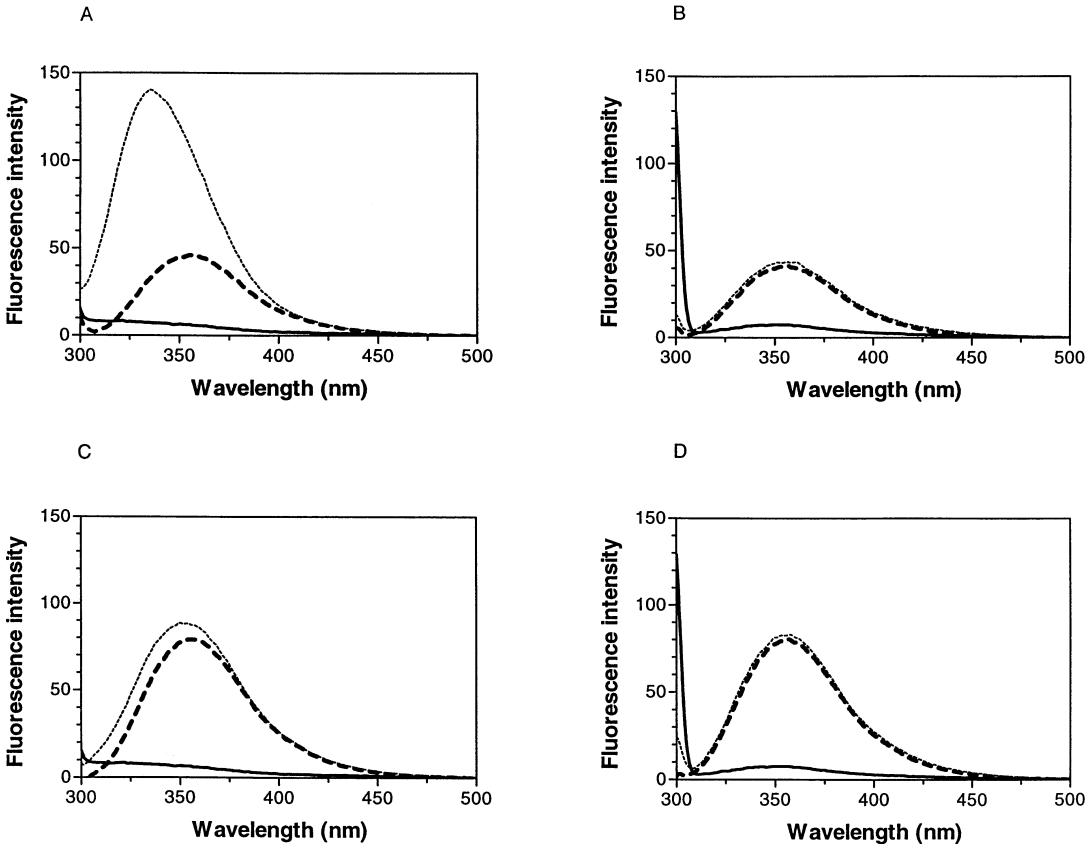

Figure 4 Tryptophan fluorescence spectra of peptide binding to CaM

Tryptophan fluorescence spectra were measured in the presence of Ca^{2+} buffer (50 mM Mops, pH 7.5, 0.1 M KCl, 0.1 mM $CaCl_2$) or in EGTA buffer (50 mM Mops, pH 7.5, 0.1 M KCl, 2 mM EGTA) using a Perkin–Elmer LS50 Luminescence Spectrometer. An excitation wavelength of 295 nm was used to reduce Tyr fluorescence from CaM. CaM (2.7 μM) was added in a solution containing 1.8 μM peptide 2 (**A** and **B**) or pAq (**C** and **D**) in the presence of 0.1 mM Ca^{2+} (**A** and **C**) or 2 mM EGTA (**B** and **D**). Thick broken line, peptide alone; thin broken line, peptide + CaM. The fluorescence spectrum of CaM alone (solid line) is shown as a control.

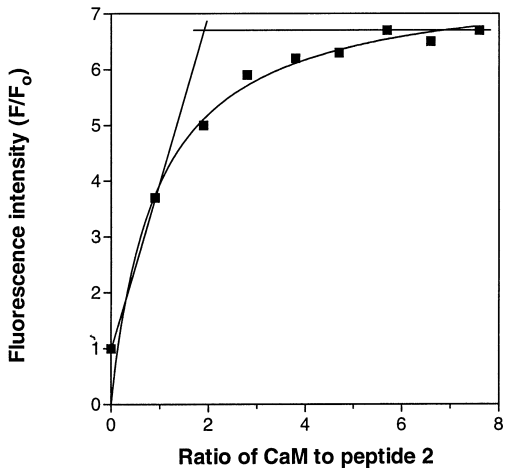

Figure 5 Stoichiometry of binding of peptide 2 to CaM

Fluorescence measurements were carried out as described in the legend to Figure 3 in the presence of Ca^{2+}. Successive 1 μl aliquots of 0.7 mM CaM, resulting in incremental 1.8 μM increases in CaM concentration, were added to 400 μl of 1.9 μM peptide 2 in the presence of Ca^{2+}. The results are expressed as the ratio of the fluorescence intensity of each sample to that of the peptide alone.

Peptide pAq, on the other hand, showed very little interaction with CaM in the presence of Ca^{2+} (Figure 4C), and no interaction in the presence of EGTA (Figure 4D).

The stoichiometry of the peptide 2–CaM complex was estimated by monitoring the total fluorescence intensity of the peptide as a function of the CaM/peptide molar ratio. Successive aliquots of CaM, resulting in an increase of 1.8 μM at a time, were added to 1.9 μM peptide 2. The fluorescence intensity ratio was proportional to the CaM/peptide 2 molar ratio until it was equal to approx. 2 (Figure 5), suggesting that CaM does not bind more than one peptide molecule at a time.

Peptide 1 and peptide 2 inhibit CaM-dependent enzyme activities selectively

The ability of peptides 1 and 2 to bind to CaM and compete with the activation of CaM kinase I, CaM kinase II and CaM PDE was investigated. Peptide 1 inhibited activation of CaM kinase I by Ca^{2+}/CaM (Figure 6A). Addition of peptide/CaM at a molar ratio of 1 resulted in $\sim 70\%$ of maximum activity; the activity decreased at higher concentrations of peptide, with $\sim 5\%$ of maximal enzyme activity remaining when peptide 1 was present in 5-fold molar excess (5 μM) compared with CaM (1 μM). Peptide 2 inhibited CaM kinase I to a much lesser extent: a 10-

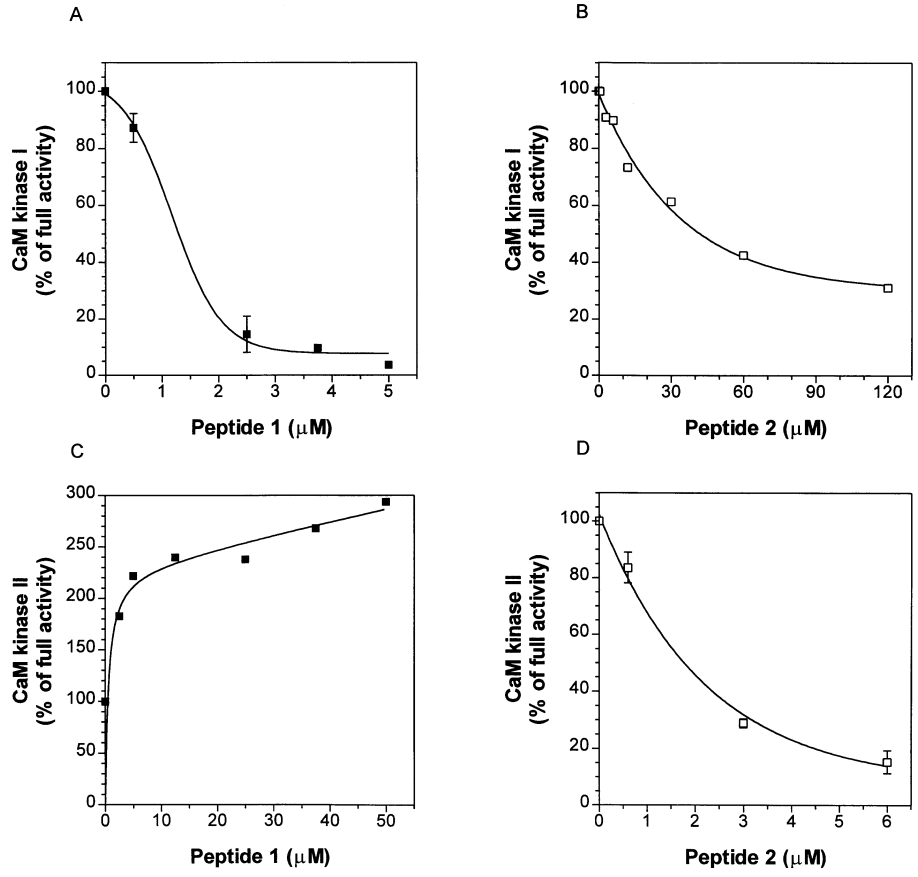

Figure 6 Effects of peptides 1 and 2 on activation of CaM kinases I and II by CaM

CaM kinase I (**A** and **B**) and CaM kinase II (**C** and **D**) activities were measured in the presence of Ca^{2+} and CaM (1 μM) with increasing concentrations of peptide 1 (**A** and **C**) or peptide 2 (**B** and **D**). Results in (**A**) and (**D**) are combined from five and three experiments respectively, and are means $\pm$ S.D. ($n = 2$–3). Results in (**B**) and (**C**) are representative of four and two experiments respectively.

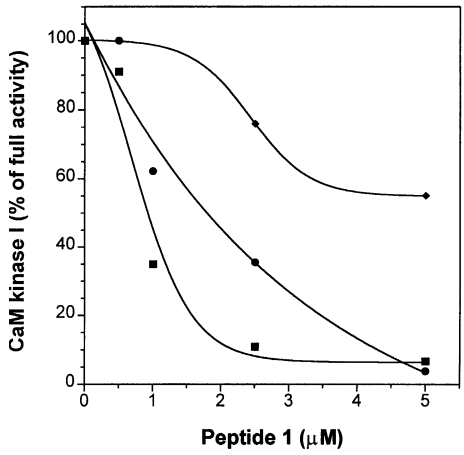

Figure 7 Effect of increasing CaM concentration on the inhibition of CaM kinase I activity by peptide 1

CaM kinase I activity was measured in the presence of Ca^{2+} and with increasing concentrations of peptide 1, and with the addition of 1 μM (■), 4 μM (●) or 11 μM (◆) CaM. Results are representative of two experiments.

fold molar excess of peptide 2 over CaM (10 μM peptide) caused only a 20% reduction in activity. However, greater inhibition was observed at higher peptide 2 concentrations: 60 μM peptide 2 inhibited CaM kinase I activity by approx. 60% and 120 μM peptide by 70% (Figure 6B). The control peptide, pAq, had no effect on CaM kinase I even at 200 μM (results not shown).

Peptide 2 inhibited CaM kinase II activity in a manner reminiscent of the effect of peptide 1 on CaM kinase I. Addition of a peptide/CaM ratio of 1 resulted in ~ 70% of full activity, and activity decreased at higher concentrations of peptide, with ~ 20% of maximal enzyme activity remaining when peptide 2 was present in a 6-fold molar excess over CaM (Figure 6D). In contrast, peptide 1 did not inhibit CaM kinase II, but rather increased the maximal activity in a concentration-dependent manner, suggesting a possible stabilizing effect on the interaction between CaM and CaM kinase II (Figure 6C). As in the case of CaM kinase I, pAq at concentrations up to 200 μM had no effect on CaM kinase II activity (results not shown). No significant differences were observed in the effects of peptides 1 and 2 on CaM kinase I or II activation when using spinach, petunia, *Xenopus*, bovine, *Neurospora* or scallop CaM (results not shown).

None of the peptides (1, 2 or pAq) inhibited CaM PDE activity, even at 60-fold molar excess over CaM (results not shown). In addition, the presence of 100 μM peptide 1 or peptide 2 had no effect on the increase in CaM PDE activity when the Ca^{2+} concentration was increased from 10 to 50 μM (results not shown).

Finally, we also examined the effects of peptides 1 and 2 on atCDPK6. Members of this family of Ca^{2+}-dependent protein kinases, which have been characterized throughout the plant kingdom, have been found to bear similarity to CaM in their regulatory domain [40]. Furthermore, as in the case of CaM, it has been suggested that binding sites for positively charged amphipathic compounds become exposed on CDPKs in response to Ca^{2+} binding [41,42]. Peptide 1 weakly inhibited the activity of atCDPK6: 40% of the maximum activity was inhibited by 160 μM peptide 1. However, peptide 2 at the same concentration had no effect on atCDPK6 (results not shown).

The NMR and fluorescence results suggested that peptides 1 and 2 bound to CaM in a Ca^{2+}-dependent fashion. However, to confirm that the observed inhibitory effect on the CaM kinases was due to binding of peptide to CaM rather than to the kinase, the effect of increasing the CaM concentration on CaM kinase I inhibition by peptide 1 was examined. The IC_{50} of peptide 1 increased in a manner proportional to the increase in CaM concentration, with values of 0.9 μM, 1.7 μM and > 5.5 μM with 1, 4 and 11 μM CaM respectively (Figure 7).

Effects of N- and C-terminal tryptic fragments of CaM on binding of peptide 1 to CaM

Two aromatic or long-chain hydrophobic amino acids, separated by 8–12 residues, have been proposed to contribute to the high-affinity binding of amphipathic α-helical target sequences by docking the peptide to the N- and C-terminal lobes of CaM [7–9]. Peptide 1 has two aromatic residues, Trp and Phe, in its N- and C-termini respectively, but they are separated by only six amino acids, suggesting that it is unlikely that the peptide would bind to both lobes of CaM. Therefore it was of interest to examine whether either the N- or the C-terminal lobe of CaM could compete with whole CaM for binding to peptide 1. Including either half of scallop CaM, even at > 60-fold molar excess as compared with whole CaM, did not alleviate inhibition of CaM kinase I by peptide 1 at CaM and peptide concentrations of 1 μM and 3–10 μM respectively. In the absence of CaM, neither fragment activated CaM kinase I above 5% of full activity (compared with the same molar concentration of CaM), even at levels as high as 62.5 μM.

DISCUSSION

In the present study we have identified, by screening of a bacteriophage library expressing random peptides, two types of phage clones enriched by binding to CaM. Based on the frequencies of the amino acid sequences obtained, two eight-amino-acid peptides, representative of the two types, were synthesized and analysed. Both peptides, termed peptide 1 (WDTVRISFG) and peptide 2 (WPSLQAIRG), were found to bind to CaM in a Ca^{2+}-dependent fashion and to differentially regulate the ability of CaM to activate CaM kinase I, CaM kinase II and CaM PDE. In addition, peptide 1, but not peptide 2, inhibited atCDPK6, albeit at high concentrations. ^1H NMR spectroscopy indicated that both peptides bound to CaM in a Ca^{2+}-dependent manner. Furthermore, binding of peptide 2 to CaM was confirmed by fluorimetry and was found to cause both increased intensity and blue-shifting of the Trp emission spectrum. These results suggest that the Trp residue in each peptide interacts with a hydrophobic environment of CaM, possibly in either or both of the two hydrophobic surfaces that are exposed in CaM upon binding of Ca^{2+}.

The two classes of CaM-binding peptides identified in the present study have certain features in common with those previously described by Dedman et al. [36]. In this previous study, a number of peptides comprising 15 amino acids were identified which bound to CaM in a Ca^{2+}-dependent manner. The most conserved feature among the sequences was Trp in the first variable position, with a second, less conserved, feature being Pro in the second variable position (11 out of 28 sequences). Our results, which were obtained using a different selection method, also placed Trp in the first variable position in peptides 1 and 2. In addition, our type 2 peptides contained Pro in the second variable position (~ 25% of all sequences). Notably, the first four variable residues of peptide 2 (WPSL) were identical with one of the 'Group 1' peptides described by Dedman et al. [36]. However, in our study, peptide 2 bound to CaM in a Ca^{2+}-

dependent manner, while a related 'truncated' peptide used as a control by Dedman et al. (AEWPSLTEIK) did not bind to CaM in a Ca^{2+}-dependent manner. Perhaps the two Glu residues in the peptide used by Dedman et al., which were not present in our peptide 2, were responsible for the lack of binding to CaM. Finally, the type 1 peptides which were most frequently isolated in the present study were not identified by Dedman et al. [36].

Peptides 1 and 2 are shorter than any peptides thus far reported to bind to CaM in a Ca^{2+}-dependent manner. In addition, they lack certain features of CaM-binding peptides that have been suggested to be critical for binding. While the primary sequences of these previously identified CaM-binding peptides have limited identity, many contain two aromatic or long-chain hydrophobic amino acids separated by 8–12 residues. The ability of CaM to accommodate different target sequences in its hydrophobic pockets is believed to be due to flexibility of the central helix and local rearrangement of side chains in the binding surface [7–9]. In many cases, Trp is found at the N-terminal end of CaM-binding peptides. Notably, both type 1 and type 2 peptides contain Trp in the first variable position; however, the type 2 peptides contain no other bulky hydrophobic residue, and the Phe found at position 8 in type 1 peptides is only separated by six residues from Trp. Thus it appears unlikely that the peptides isolated could bind simultaneously to both the N- and C-terminal lobes of CaM. Peptides derived from CaM-binding sites of target proteins that are large enough to span the length of the hydrophobic binding surface of CaM and bind to both lobes simultaneously have been found to be general CaM inhibitors [26,43]. For example, CaM-binding peptides derived from CaM kinase II, and skeletal or smooth muscle myosin light chain kinase, were found to inhibit CaM PDE with the same efficacy. Therefore the fact that representatives of the two types of peptide identified in the present study differentially inhibited CaM-dependent enzymes supports the conclusion that the peptides do not bind to both lobes of CaM at the same time.

The precise nature of the interactions of the peptides cannot be determined from our present studies. Binding of peptides 1 and 2 was found to induce similar changes in the ^1H NMR spectrum of CaM, despite the differences in their sequences. In addition, while it is not possible to make any quantitative evaluation, the ^1H NMR results suggest that peptides 1 and 2 interact with CaM in a manner distinct (results not shown) from that of the known high-affinity amphipathic α-helical peptide of skeletal muscle myosin light chain kinase [7]. Thus it is possible that the peptides could bind in a Ca^{2+}-dependent manner to either the N- or the C-terminal domain alone of CaM. Alternatively, although not supported by the stoichiometry results for peptide 2, either peptide could bind to both N- or C-terminal domains of CaM, as has been suggested for the CaM-binding peptide mastoparan [22,27].

Peptides 1 and 2 described in this study have the unique ability to be able to differentially influence the CaM-dependent activation of two CaM kinases and CaM PDE. The basis for these effects may be related to the precise nature of the interaction of Ca^{2+}/CaM with different CaM-dependent enzymes. As discussed in the Introduction, a number of studies have suggested that CaM does not interact in the same way with its different targets. In addition, the primary structures of the CaM-binding domains of various target enzymes are not highly conserved [6,10]. For example, the CaM-binding domain of CaM kinase I includes the sequence KWKQAFNATAVVRHMRK [44], with Trp^{303} in CaM kinase I aligning with the conserved Trp in the CaM-binding domains of skeletal muscle and smooth muscle myosin light chain kinases [7]. In contrast, the CaM-binding domain of CaM kinase II, NARRKLKGAILTTMLATR, does not contain a conserved Trp residue, but instead contains two Leu residues, eight amino acids apart, that interact respectively with the C- and N-terminal lobes of CaM [9]. Furthermore, CaM kinase II is a multi-subunit enzyme that displays complex regulation of CaM binding in response to autophosphorylation [45]. Thus, while there appear to be common features in the interactions of CaM-regulated enymes with CaM, the differential effects of peptides 1 and 2 may be related to the differences found in the specific regions of CaM that interact with each target enzyme, or to the differences in the region of each target enzyme that interacts with CaM.

Without assuming that any specific structural subdomain of CaM is responsible for the interaction with the CaM-binding domains of CaM kinase I, CaM kinase II or CaM PDE, a simple model that would explain our results is possible if there are minimally two domains of CaM that are differentially involved in activating target enzymes. Furthermore, the model would predict that both domains 1 and 2 of CaM would be necessary for binding to and activation of CaM kinase I, that only domain 2 of CaM would be necessary for binding to and activation of CaM kinase II, and that either domain 1 or domain 2 of CaM would be sufficient for binding to and activation of CaM PDE. If this were the case, peptide 1 may be predicted to bind only to domain 1, leading to incomplete activation of CaM kinase I but not CaM kinase II. In contrast, peptide 2 may be predicted to bind only to domain 2, but in this case the activation of both CaM kinases I and II would be affected. However, until three-dimensional structural information is available, this type of explanation for our present results, as well as previous results pertaining to CaM and the activation of its target enzymes, clearly will remain speculative.

In conclusion, two novel CaM-binding peptides have been identified that exhibit distinct abilities to influence the activation of CaM-dependent enzymes by CaM. The properties of these two peptides may reflect their abilities to interact with different subdomains of CaM, although other explanations may be possible. The unique inhibitory effects of peptides 1 and 2 may be related to their short length, which prevents them from binding to CaM in the same way as previously described CaM-binding peptides that act as general inhibitors of CaM function. The information obtained from the present study will hopefully allow further design of different peptide analogues that may act as more specific inhibitors of distinct CaM-dependent enzymes. Alternatively, screening of peptide libraries of the type used in the present study may help in the identification of specific inhibitors of the interactions of various target proteins with CaM. This, in turn, may provide reagents that will be valuable in determining the physiological functions of this important class of enzymes.

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REFERENCES

1 Davis, T. N. (1992) Cell **71**, 557–564
2 Hanson, P. I. and Schulman, H. (1992) Annu. Rev. Biochem. **61**, 559–601
3 Lu, K. P. and Means, A. R. (1993) Endocr. Rev. **14**, 40–58
4 Nairn, A. C. and Picciotto, M. R. (1994) Semin. Cancer Biol. **5**, 295–303
5 Klee, C. B. (1988) in Molecular Aspects of Cellular Regulation (Cohen, P. and Klee, C. B., eds.), vol. 5, pp. 35–56, Elsevier, Amsterdam

6 O'Neil, K. T. and DeGrado, W. F. (1990) Trends Biochem. Sci. **15**, 59–64
7 Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B. and Bax, A. (1992) Science **256**, 632–638
8 Meador, W. E., Means, A. R. and Quiocho, F. A. (1992) Science **257**, 1251–1255
9 Meador, W. E., Means, A. R. and Quiocho, F. A. (1993) Science **262**, 1718–1721
10 Afshar, M., Caves, L. S., Guimard, L., Hubbard, R. E., Calas, B., Grassy, G. and Haiech, J. (1994) J. Mol. Biol. **244**, 554–571
11 Putkey, J. A., Draetta, G. F., Slaughter, G. R., Klee, C. B., Cohen, P., Stull, J. T. and Means, A. R. (1986) J. Biol. Chem. **262**, 9896–9903
12 Hurwitz, M. Y., Putkey, J. A., Klee, C. B. and Means, A. R. (1988) FEBS Lett. **238**, 82–86
13 Mann, D. M. and Vanaman, T. C. (1989) J. Biol. Chem. **264**, 2373–2378
14 Persechini, A., Blumenthal, D. K., Jarrett, H. W., Klee, C. B., Hardy, D. O. and Kretsinger, R. H. (1989) J. Biol. Chem. **264**, 8052–8058
15 George, S. E., VanBerkum, M. F. A., Ono, T., Cook, R., Hanley, R. M., Putkey, J. A. and Means, A. R. (1990) J. Biol. Chem. **265**, 9228–9235
16 Chabbert, M., Lukas, T. J., Watterson, M., Axelsen, P. H. and Prendergast, F. G. (1991) Biochemistry **30**, 7615–7630
17 Haiech, J., Kilhoffer, M.-C., Lukas, T. J., Craig, T. A., Roberts, D. M. and Watterson, D. M. (1991) J. Biol. Chem. **266**, 3427–3431
18 Bagchi, I. C., Huang, Q. and Means, A. R. (1992) J. Biol. Chem. **267**, 3024–3029
19 Gao, Z. H., Krebs, J., VanBerkum, M. F. A., Tang, W.-J., Maune, J. F., Means, A. R., Stull, J. T. and Beckingham, K. (1993) J. Biol. Chem. **268**, 20096–20104
20 George, S. E., Su, Z., Fan, D. and Means, A. R. (1993) J. Biol. Chem. **268**, 25213–25220
21 Farrar, Y. J. K., Lukas, T. J., Craig, T. A., Watterson, D. M. and Carlson, G. M. (1993) J. Biol. Chem. **268**, 4120–4125
22 Mukherjea, P. and Beckingham, K. (1993) Biochem. Mol. Biol. Int. **29**, 555–563
23 Persechini, A., McMillan, K. and Leakey, P. (1994) J. Biol. Chem. **269**, 16148–16154
24 Su, Z., Fan, D. and George, S. E. (1994) J. Biol. Chem. **269**, 16761–16765
25 Zhang, M., Li, M., Wang, J. H. and Vogel, H. J. (1994) J. Biol. Chem. **269**, 15546–15552
26 Hanley, M. M., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxham, N. and Kelly, P. T. (1987) Science **237**, 293–297
27 Kataoka, M., Head, J. F., Seaton, B. A. and Engelman, D. M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6944–6948
28 Yoshino, H., Minari, O., Matsuhima, N., Ueki, T., Miyake, Y., Matsuo, T. and Izumi, Y. (1989) J. Biol. Chem. **264**, 19706–19709
29 Picciotto, M. R., Czernik, A. J. and Nairn, A. C. (1993) J. Biol. Chem. **268**, 26512–26521
30 McGuinness, T. L., Lai, Y. and Greengard, P. (1985) J. Biol. Chem. **260**, 1696–1704
31 Scott, J. K. and Smith, G. P. (1990) Science **249**, 386–390
32 Yanish-Perron, C., Vieira, J. and Messing, J. (1985) Gene **33**, 103–119
33 Takeshita, S., Sato, M., Toba, M., Masahashi, W. and Hashimoto-Goto, T. (1987) Gene **61**, 63–74
34 Kunkel, T. A., Robert, J. D. and Zakour, R. A. (1987) Methods Enzymol. **154**, 367–382
35 Cwirla, S. E., Peters, E. A., Barrett, R. W. and Dower, W. J. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6378–6382
36 Dedman, J. R., Kaetzel, M. A., Cham, H. C., Nelson, D. J. and Jaimieson, Jr., G. A. (1993) J. Biol. Chem. **268**, 23025–23030
37 Charbonneau, H., Kumar, S., Novack, J. P., Blumenthal, D. K., Griffin, P. R., Shabanowitz, J., Hunt, D. F., Beavo, J. A. and Walsh, K. A. (1991) Biochemistry **30**, 7931–7940
38 Minowa, O. and Yagi, K. (1989) J. Biochem. (Tokyo) **96**, 1251–1255
39 Ikura, M., Hiraoki, T., Hikichi, K., Mikuni, T., Yazawa, M. and Yagi, K. (1983) Biochemistry **22**, 2568–2572
40 Roberts, D. M. (1993) Curr. Opin. Cell Biol. **5**, 242–246
41 Harper, J. F., Binder, B. M. and Sussman, M. R. (1993) Biochemistry **32**, 3282–3290
42 Polya, G. M., Nott, R., Klucis, E., Minichello, J. and Chandra, S. (1990) Biochim. Biophys. Acta **1037**, 259–262
43 Graff, J. M., Rajan, R. R., Randall, R. R., Nairn, A. C. and Blackshear, P. J. (1991) J. Biol. Chem. **266**, 14390–14398
44 Yokokura, H., Picciotto, M. R., Nairn, A. C. and Hidaka, H. (1995) J. Biol. Chem. **270**, 23851–23859
45 Braun, A. P. and Schulman, H. (1995) Annu. Rev. Physiol. **57**, 417–445

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