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Domain II of calmodulin is involved in activation of calcineurin

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A family of mutant proteins related to calmodulin (CaM) has been produced using cDNA constructs in bacterial expression vectors. The new proteins contain amino acid substitutions in Ca^{2+}-binding domains I, II, both I and II, or both II and IV. The calmodulin-like proteins have been characterized with respect to mobility on SDS-polyacrylamide gels, Ca^{2+}-dependent enhancement of tyrosine fluorescence, and abilities to activate the CaM-dependent phosphatase calcineurin. These studies suggest that an intact Ca^{2+}-binding domain II is minimally required for full activation of calcineurin.

Calmodulin; Calcineurin; Mutagenesis

1. INTRODUCTION

Calmodulin (CaM) regulates the activity of a large number of enzymes. Studies using chemically-modified [1–7] or site-specifically mutated [8–12] CaMs have revealed that individual enzymes are differentially affected by any given alteration. The protein phosphatase calcineurin is only partially activated by a CaM to which a phenothiazine has been covalently attached [13]. Similarly calmodulin-like (CaML) proteins (CaML16 and CaML19) have been described that can activate calcineurin only partially but would maximally activate cGMP phosphodiesterase (PDE) or myosin light-chain kinase (MLCK) [9]. CaML proteins are the bacterially produced product of a chicken calmodulin pseudogene that contains 19 amino acid substitutions relative to calmodulin [14]. These substitutions exist in Ca^{2+}-binding domains I, II and IV. In order to define the domain containing the substitutions that results in a protein with partial agonist activity relative to calcineurin, a family of new proteins has been produced by combining portions of the pseudogene and two CaM cDNAs. The new CaML proteins contain amino acid substitutions in Ca^{2+} binding domains I, II, both I and II, or both II and IV. Analysis of these proteins shows that domain II contains the mutations that result in reduced ability to act as an agonist of calcineurin.

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2. EXPERIMENTAL

2.1. *Construction of bacterial expression vectors for CaML proteins*

The expression plasmids for the new CaML proteins were constructed from previously described cDNAs for normal eel CaM (pCM116) [15], normal chicken CaM (pcB12) [16], a chicken processed CaM pseudogene (CM1) [17], and the expression plasmid for CM1 (pCaML19) [9]. Enzymes were obtained from Bethesda Research Laboratories and were used according to the manufacturer's recommendations. DNA fragments were isolated using either low-melting agarose or 5% PAGE as described below.

2.1.1. Construction of pCaML13

Ca^{2+}-binding domain I was isolated from pCM116 as the 138 base-pair (bp) *Sau*3A/*Pst*I subfragment of the 171 bp *Pst*I fragment. Ca^{2+}-binding domains II and III were isolated from pCaML16. pCaML16 was linearized with *Pst*I,

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dephosphorylated with bacterial alkaline phosphatase (BAP) and then digested with *Bcl*I to yield a 229 bp fragment. Using T_4 DNA ligase, these two small fragments were ligated into a BAP-treated 3232 bp *Bcl*I fragment of pCaML16. The 3232 bp fragment contains Ca^{2+}-binding domain IV in a pUC8 vector [8].

2.1.2. Construction of pCaML3A

A 712 bp *Pst*I fragment of pCM116 which contained the Ca^{2+}-binding domains II, III, and IV was ligated into pCaML16 which had been linearized with *Pst*I and treated with BAP. The pCaML16 contributed Ca^{2+}-binding domain I in a pUC8 vector.

2.1.3. Construction of the intermediate pCaML13A

A 121 bp *Sau*3A subfragment of a 400 bp *Alu*I fragment of pCM116 containing Ca^{2+}-binding domain IV was ligated into pCaML19 which had been linearized with *Bcl*I and dephosphorylated with calf intestinal phosphatase (CIP). pCaML19 is in the vector pBR322 and contributes the Ca^{2+}-binding domains I, II, and III to the new coding region.

2.1.4. Construction of pCaML10

A 154 bp *Pst*I/*Bam*HI fragment containing Ca^{2+}-binding domains I and II of pCaML16 was ligated into the large *Pst*I/*Bam*HI fragment of pCaML13A. pCaML13A contributed Ca^{2+}-binding domains III and IV.

2.1.5. Construction of pCaML7

A 154 bp *Pst*I/*Bam*HI fragment containing the first two Ca^{2+}-binding domains of pCaML13 was ligated into the large *Pst*I/*Bam*HI fragment of pCaML13A. pCaML13A contributed the last two Ca^{2+}-binding domains.

2.2. *Purification of bacterially expressed CaM proteins*

Proteins were purified from bacterial lysates using differential chromatography on phenyl-Sepharose in the presence and absence of Ca^{2+} as described [8]. The proteins were further purified using HPLC. Samples were applied to a TSK-DEAE-5PW (BioRad) column (75 mm × 7.5 cm) and then eluted with a linear gradient from 0–0.5 M NaCl in 10 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM EDTA.

2.3. *Electrophoresis*

Electrophoresis of DNA fragments was performed either on 1% low-melting point agarose or 5% PAGE [8]. DNA fragments were isolated using 'NACS' prepacked columns according to the manufacturer's recommendations (Bethesda Research Laboratories).

Protein electrophoresis was performed on 15% SDS-PAGE according to the method of Laemmli [18]. The protein samples were solubilized in sample buffer containing either 5 mM $CaCl_2$ or 5 mM EDTA.

2.4. *Tyrosine fluorescence*

Tyrosine fluorescence was measured using an Aminco SPF-500 Ratio Spectrofluorometer. Solutions containing 0.2 mg protein per ml 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2 mM EGTA (22°C) were excited at 275 nm and fluorescent emission was monitored at 307 nm. Ca^{2+} solutions were prepared by dilution from Orion standard Ca^{2+} solution (0.1 M). Free Ca^{2+} concentrations were calculated using a computer program developed by Goldstein [19].

2.5. *Calcineurin assays*

Calcineurin was prepared and assayed as described by Newton et al. using *p*-nitrophenyl phosphate as the substrate [20].

3. RESULTS AND DISCUSSION

The schemes for the construction of the four new expression plasmids (pCaML3A, pCaML7, pCaML10, and pCaML13) are described in section 2 and shown in fig.1. The resulting plasmids were used to transform the *E. coli* strain JM103. The structures of the newly constructed CaML cDNAs were confirmed by restriction and sequence analysis. The location of amino acid sequence differences between the proteins encoded by the newly constructed CaML cDNAs and pCaM23 are indicated by the open (conservative changes) and closed (non-conservative changes) circles along the bar diagrams in fig.1. Conforming to the nomenclature introduced by Putkey et al. [9], the CaML plasmids and their corresponding protein products are named to indicate the number of amino acid differences with respect to normal chicken CaM.

For each plasmid, 6 l of bacterial culture grown in the presence of the inducer isopropyl-β-D-thiogalactoside (IPTG) were used as the source of the bacterially synthesized proteins. Protein purifications were performed as described in section 2 and yielded >30 mg of each CaM or CaML protein. SDS-PAGE was performed in the presence or absence of Ca^{2+}. The gel in fig.2 shows the relative mobilities of CaM23 and of the CaML proteins. In the absence of Ca^{2+}, all the proteins co-migrated with CaM23. CaM23 and CaML7 are shown as representative proteins. In the presence of Ca^{2+}, all the proteins demonstrated an ion-dependent shift in mobility. The extent of the induced migration shift, however, is related to the number of amino acid substitutions in the CaML protein. CaM23 and CaML3A which has only three amino acid substitutions in Ca^{2+}-binding domain I show the greatest shifts. CaML16 and CaML13 show the least effect of Ca^{2+} on the migration of SDS-PAGE, and CaML10 and CaML7 show an intermediate effect.

CaM shows a characteristic enhancement of

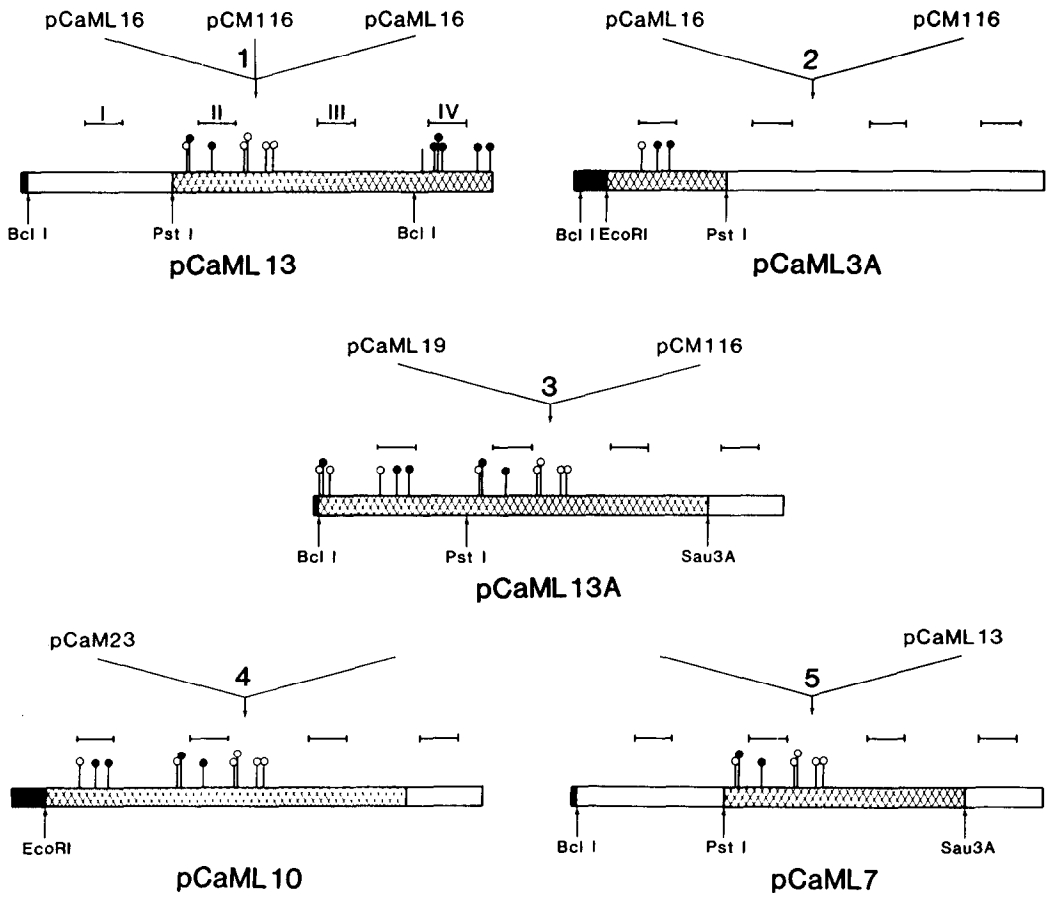

Fig.1. Construction of pCaML expression plasmids. Expression vectors for calmodulin-like proteins were constructed as described in section 2. The amino acid substitutions indicated by the open (conservative) and closed (non-conservative) circles are: domain I – $K^{21} \rightarrow R$, $T^{26} \rightarrow C$, and $K^{30} \rightarrow M$; domain II – $I^{52} \rightarrow V$, $N^{53} \rightarrow G$, $N^{60} \rightarrow S$, $T^{70} \rightarrow S$, $M^{71} \rightarrow L$, $K^{77} \rightarrow R$, and $T^{79} \rightarrow S$; domain IV – $R^{126} \rightarrow K$, $I^{130} \rightarrow C$, $D^{131} \rightarrow N$, $G^{132} \rightarrow R$, and $A^{147} \rightarrow Q$.

tyrosine fluorescence emission at 307 nm when CaM is excited at 275 nm in the presence of Ca^{2+} vs in the absence of Ca^{2+} [21]. Fig.3 shows the ratio of the relative fluorescence in the presence of Ca^{2+} to the relative fluorescence in the absence of Ca^{2+} as a function of free Ca^{2+} concentration. The curve derived for CaM23 is identical to the curves derived for CaML3A, CaML7, and CaML10 (not shown). Those curves, however, are distinctly different from the ones generated for CaML13 and CaML16, both of which contain amino acid substitutions in Ca^{2+}-binding domain IV. The reduction of the Ca^{2+}-induced enhancement of tyrosine over physiologic ranges of Ca^{2+} concentrations appears to be specifically related to changes in the amino acid sequence in Ca^{2+}-binding domain IV. This reduction is probably due to a change in the microenvironment around tyrosine 138 (domain IV) since Ca^{2+} binding seems to affect this microenvironment selectively [21].

The activation of calcineurin by the CaML proteins showed a dependence on an intact Ca^{2+}-binding domain II. As shown in fig.4, CaML3A fully activates calcineurin as compared to CaM23. However, any CaML protein containing the seven amino acid substitutions in Ca^{2+}-binding domain II showed a significant decrease in the ability of the protein to activate calcineurin. CaML7, 10, 13, and 16 activate

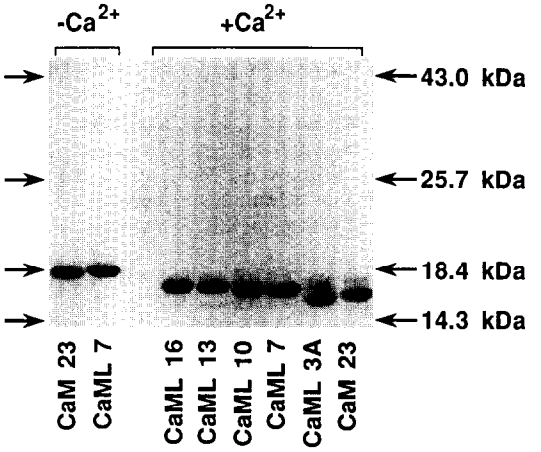

Fig.2. SDS-PAGE of CaML proteins in the presence and absence of Ca^{2+}.

calcineurin about 35–50% as well as CaM23. *P* values for these four proteins are <0.001 with respect to CaM23 but >0.02 with respect to each other. In contrast, none of the CaML proteins show changes in their abilities to maximally activate PDE or MLCK (not shown).

CaM is a complex regulatory protein which is instrumental in transmitting intracellular signals through Ca^{2+}-dependent second messenger pathways. The protein binds Ca^{2+} and this complex specifically interacts with numerous different proteins thereby inducing activity changes. In order to understand how CaM can perform this regulatory function efficiently, the specificity of the interactions between CaM and the CaM-binding proteins must be defined. Although changes in the amino acid sequence in Ca^{2+}-binding domains I, II, or IV are reflected in some of the Ca^{2+}-dependent physical characteristics of the molecule, they do not affect the ability of the proteins to activate PDE or MLCK. In contrast, amino acid substitutions in a single Ca^{2+}-binding domain (II) are sufficient to alter the ability of CaM to activate calcineurin significantly. CaML7 contains the following amino acid substitutions: $I^{52} \longrightarrow V$; $N^{53} \longrightarrow G$; $N^{60} \longrightarrow S$; $T^{70} \longrightarrow S$; $M^{71} \longrightarrow L$; $K^{77} \longrightarrow R$ and $T^{79} \longrightarrow S$. From analysis of the crystal structure of CaM at 2.2 Å [22] the most severe structural changes would be predicted to be $N^{53} \longrightarrow G$ and $K^{77} \longrightarrow R$. Site specific mutagenesis can now be employed to provide a definite answer.

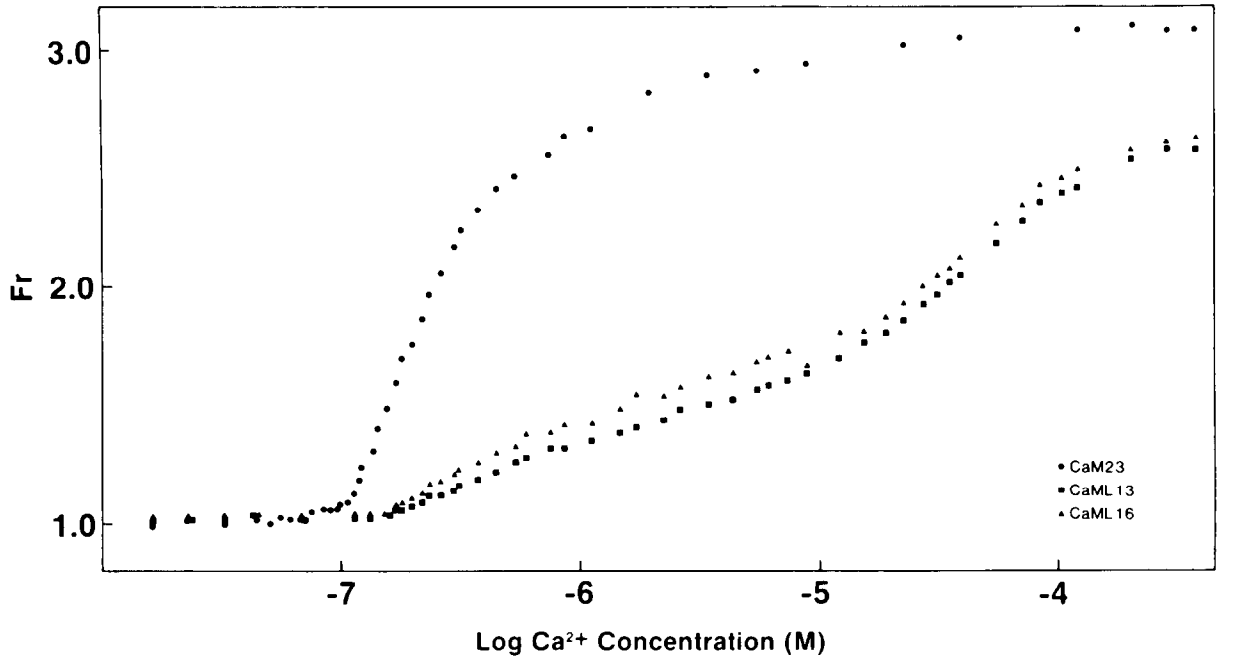

Fig.3. Ca^{2+}-dependence of enhanced tyrosine fluorescence. F_r is the ratio of the relative fluorescence in the presence of Ca^{2+} to the relative fluorescence in the absence of Ca^{2+}.

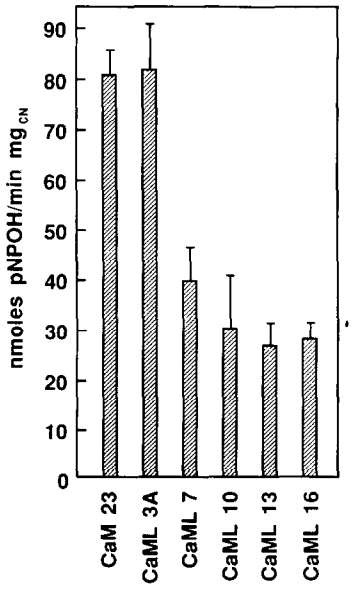

Fig.4. Activation of calcineurin by CaML proteins. Each bar represents the average of four representative independent experiments.

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