Characterization of the Human Calmodulin-like Protein Expressed in *Escherichia coli*†

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ABSTRACT: The protein-coding region of an intronless human calmodulin-like gene [Koller, M., & Strehler, E. E. (1988) *FEBS Lett. 239*, 121–128] has been inserted into a pKK233-2 expression vector, and the 148-residue, M_r = 16 800 human protein was purified to apparent homogeneity by phenyl-Sepharose affinity chromatography from cultures of *Escherichia coli* JM105 transformed with the recombinant vector. Several milligrams of the purified protein were obtained from 1 L of bacterial culture. A number of properties of human CLP were compared to those of bacterially expressed human calmodulin (CaM) and of bovine brain CaM. CLP showed a characteristic Ca^{2+}-dependent electrophoretic mobility shift on SDS–polyacrylamide gels, although the magnitude of this shift was smaller than that observed with CaM. CLP was able to activate the 3′,5′-cyclic nucleotide phosphodiesterase to the same V_{max} as normal CaM, albeit with a 7-fold higher K_{act}. In contrast, the erythrocyte plasma membrane Ca^{2+}-ATPase could only be stimulated to 62% of its maximal CaM-dependent activity by CLP. CLP was found to contain four Ca^{2+}-binding sites with a mean affinity constant of 10^5 M^{-1}, a value about 10-fold lower than that for CaM under comparable conditions. The highly tissue-specifically-expressed CLP represents a novel human Ca^{2+}-binding protein showing characteristics of a CaM isoform.

Calmodulin (CaM)[1] is a ubiquitous and highly versatile intracellular high-affinity Ca^{2+}-binding protein responsible for mediating the Ca^{2+} signal to a multitude of different enzyme systems. CaM is found in all eukaryotes including protozoa, fungi, plants, and animals, and it has been highly conserved throughout evolution in plants and animals (Means et al., 1982; Klee & Vanaman, 1982). Except for the sea urchin *Arbacia punctulata* (Hardy et al., 1988) where 2 iso-CaMs differing in at least 2 (but very likely in 4) out of their 148 residues have been detected, only a single CaM protein has previously been thought to exist within a given species. Protein data suggest that iso-CaMs exist in *Naegleria flagellates* (Fulton et al., 1986), but no primary amino acid sequence data are as yet available to support this conclusion.

On the gene level, a multigene family of three maximally divergent members is responsible—at least in mammals—for the generation of the single CaM protein (Fischer et al., 1988; Nojima, 1989). In the rat and human genomes, a number of pseudogenes have also been characterized which are clearly nonfunctional as judged from the presence of internal deletions, insertions, and nonsense mutations that preclude the production of a functional protein (Nojima & Sokabe, 1986, 1987; Nojima, 1989; SenGupta et al., 1989; Koller et al., 1991). One intronless chicken gene (Stein et al., 1983) and a rat (Nojima & Sokabe, 1986) CaM-like gene have, however, retained the potential to encode a 148 and a 146 amino acid residue protein, respectively, each displaying 19 amino acid changes with respect to the known higher vertebrate CaM. More recently, a similar CaM-like gene has also been isolated from the human genome (Koller & Strehler, 1988). This intronless gene has retained the potential to encode a protein identical in length, and with a considerable degree of identity (85%), to the "bona fide" CaM. Previous efforts to detect mRNA expression of either the chicken (Putkey et al., 1987), the rat (Nojima et al., 1987), or the human CaM-like gene (Koller & Strehler, 1988) have met with no success, leading to the conjecture that these genes probably correspond to nonfunctional retropseudogenes.

Taking an entirely different approach, Yaswen et al. (1990) have succeeded in cloning a number of cDNAs expressed in normal human mammary epithelial cells but drastically underrepresented in corresponding tumor tissues. Interestingly, one of these unique cDNAs corresponds precisely to the transcribed CaM-like gene previously identified by us (Koller & Strehler, 1988). This finding demonstrates that the human CaM-like gene is functional and poses questions as to the identity, structure, and possible function of the encoded protein. In this report, we describe the overexpression of the human CaM-like protein (CLP) in *Escherichia coli*, the purification of this protein to apparent homogeneity, and its biochemical characterization and Ca^{2+}-binding properties as well as experiments that show that it stimulates the cyclic nucleotide phosphodiesterase to the same V_{max} as normal CaM (albeit with a higher K_m) whereas the human erythrocyte plasma membrane Ca^{2+}-ATPase can only be stimulated to 62% of the V_{max} achieved with CaM. The results indicate that CLP is a novel Ca^{2+}-binding protein that may act as a CaM "isoform" in specific human tissues.

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[1] Abbreviations: βME, β-mercaptoethanol; CaM, calmodulin; CLP, calmodulin-like protein; IPTG, isopropyl β-D-thiogalactopyranoside; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; PMSF, phenylmethanesulfonyl fluoride.

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MATERIALS AND METHODS

Construction of Expression Plasmids. To obtain expression plasmid pKK-CaMIII, the 469 bp *Sma*I restriction fragment containing the coding region of CaMIII cDNA [nucleotides 92–559 of the published sequence (Fischer et al., 1988); see also corrections in Koller et al. (1990)] was first subcloned into pSP65 (Promega Corp., Madison, WI). pSP65-CaMIII plasmid DNA carrying the insert in the desired orientation was digested with *Nco*I (cuts at the initiator Met codon) and *Hin*dIII (cuts in the pSP65 polylinker), and the resulting approximately 500 bp restriction fragment was ligated into *Nco*I- and *Hin*dIII-digested pKK233-2 (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). To obtain expression plasmid pKK-CLP, a 706 bp *Sma*I fragment containing the sequence encoding CLP [positions −202 to +504 of the sequence in Koller and Strehler (1988)] was first subcloned into pUC18. A 525 bp restriction fragment was obtained by digesting an appropriate pUC-CLP plasmid with *Pvu*II (cuts nine nucleotides downstream of the ATG start codon) and *Hin*dIII (cuts in the pUC polylinker). A synthetic *Nco*I blunt-end linker was synthesized (consisting of the 13-mer oligoexynucleotide 5′-CATGGCCGACCAG-3′ and the complementary 9-mer 5′-CTGGTCGGC-3′) and was ligated to the *Pvu*II end of the 525 bp restriction fragment. The synthetic linker recreates the codons for the four N-terminal amino acid residues of CLP. After restriction with *Nco*I and *Hin*dIII, the resulting 538 bp restriction fragment was ligated into pKK233-2 doubly-digested with *Nco*I and *Hin*dIII. Figure 1 shows schematic representations of the final bacterial expression plasmids for human CaM and CLP. The integrity of the constructs was confirmed by restriction enzyme mapping and nucleotide sequencing (Sanger et al., 1977) using the Sequenase Version 2.0 sequencing kit (United States Biochemical Corp., Cleveland, OH) according to the manufacturers' instructions.

Protein Expression and Purification. Bacterial expression and purification of human CaM and CLP were performed according to a modification of the method described by Putkey et al. (1986). Briefly, 1 L of 2 × YT medium containing 50 μg/mL ampicillin (Maniatis et al., 1982) was inoculated with 20 mL of an overnight culture of *E. coli* JM105 carrying the appropriate expression plasmid, and the culture was shaken at 37 °C for 2–4 h until $A_{550nm} \approx 0.4$. IPTG was added to 1.5 mM, and cell growth was continued at 37 °C for 5–7 h. Cells were harvested by centrifugation (8000*g*, 10 min, 4 °C), resuspended in 200 mL of 50 mM Tris-HCl (pH 7.5)/0.5 mM PMSF, and pelleted as above. The washed cell pellet could be stored at −70 °C or was directly resuspended in 80 mL of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM βME, and 0.5 mM PMSF. Lysozyme was added to 0.2 mg/mL, and the suspension was incubated on ice for 30 min. Four milligrams of DNase I (Sigma Chemical Co., St. Louis, MO), dissolved in 1.2 mL of 0.2 M $MgCl_2$, was added to the lysate, and the incubation was continued for 2 h on ice. The lysate was cleared by centrifugation (27000*g*, 30 min, 4 °C), and EGTA was added to the supernatant to 5 mM. Five milliliters of preswollen phenyl-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) was added, and the mixture was rotated end-over-end for 2–3 h at room temperature to allow adsorption of unspecific binding proteins. The phenyl-Sepharose was removed by centrifugation (1500*g*, 10 min), and $CaCl_2$ was added to the supernatant to a final concentration of 15 mM. This solution was passed twice over a phenyl-Sepharose CL-4B column (approximately 5-mL bed volume) equilibrated in 50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 0.5 mM βMe, and 0.5 mM PMSF. The column was washed first with 100 mL of the above buffer and then with 100 mL of the same buffer containing 500 mM NaCl. Elution of bound protein was performed with 6 mL of 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 0.5 mM βME, and 0.5 mM PMSF. The pooled protein fractions were dialyzed overnight against 3 × 2 L of 10 mM NH_4HCO_3 and lyophilized for storage. Typically, the yields of protein were 6 mg for CaM and 2 mg for CLP per liter of bacterial culture. Bovine brain CaM was purified as described by Guerini et al. (1984).

Protein Analysis. Proteins were analyzed by one-dimensional SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). For partial amino acid sequence analysis, purified CLP was cleaved by CNBr, the cleavage products were separated by reversed-phase HPLC, and sequence analysis was performed on an Applied Biosystems 470A sequencer with on-line 120A phenylthiohydantoin detection as described (James et al., 1989). Protein concentrations were measured spectrophotometrically by using the molar extinction coefficients $\epsilon_{276nm} = 3300\ M^{-1}$ for CaM and $\epsilon_{276nm} = 1500\ M^{-1}$ (plant CaM) for CLP (Klee & Vanaman, 1982). CLP, like plant CaM, contains only a single tyrosine residue (Tyr^{138}), which results in an about 2-fold lower molar extinction coefficient for these proteins than for vertebrate CaM. Concentrations of standard solutions were determined by amino acid analysis. Alternatively, CLP concentrations were determined by the modified Lowry method (Ausubel et al., 1987), using bovine brain CaM as standard.

Electrophoretic Shift. The Ca^{2+}-dependent electrophoretic mobility shift was demonstrated by SDS–polyacrylamide gel electrophoresis essentially as described by Burgess et al. (1980) except that the Ca^{2+}-free samples were boiled for 3 min in sample buffer (Laemmli, 1970) containing 5 mM EGTA before being loaded onto the gel. The electrophoresis buffer for Ca^{2+}-free conditions contained 8 mM EDTA, and that for gels in the presence of Ca^{2+} contained 0.1 mM $CaCl_2$.

Ca^{2+}-Binding Studies. For removal of contaminating metal ions and for complete equilibration of the protein in the assay buffer, CLP was precipitated with 3% trichloroacetic acid in the presence of 1 mM EDTA and then passed through a 40 × 1 cm Sephadex G-25 column equilibrated in the assay buffer. The latter was freed of contaminating metals by passage over a column of immobilized EDTA (Haner et al., 1984). Ca^{2+} contamination was always less than 0.1 mol/mol of protein. Total Ca^{2+} concentrations were determined with a Perkin-Elmer Cetus Instruments 2380 atomic absorption spectrophotometer. Ca^{2+} binding to CLP in the presence of 2 mM Mg^{2+} was measured at 25 °C by the flow dialysis method of Colowick and Womack (1969) in 30 mM Hepes, 30 mM imidazole buffer, pH 7.5, 2 mM $MgCl_2$, and 7.5 mM β-ME or in 50 mM Tris-HCl buffer, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$, and 7.5 mM β-ME. The protein concentration was 40–70 μM. Treatment of the raw data was as described by Cox et al. (1990) and Krause et al. (1991). The binding data were analyzed by means of the Adair (1925) equation for four binding sites, and the stoichiometric binding constants (K) were calculated iteratively with a curve-fitting computer program, i.e., Simplex in the Matlab environment. The intrinsic association constants (K') were obtained by applying the statistical factors 1/4, 2/3, 3/2, and 4 to the stoichiometric binding constants K_1, K_2, K_3, and K_4, respectively (Cornish-Bowden & Koshland, 1975).

Phosphodiesterase Assay. The activity of cyclic nucleotide phosphodiesterase from bovine heart was determined by

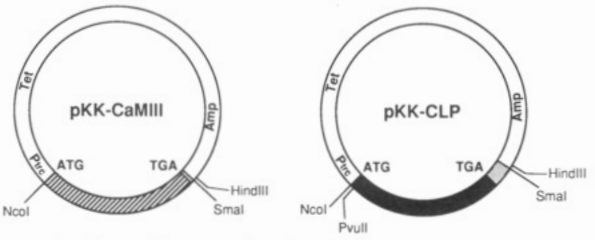

FIGURE 1: Bacterial expression plasmids for CaM and CLP. Open regions, plasmid pKK233-2; hatched region, coding sequence of the human CaMIII cDNA; black region, coding sequence of the human CLP gene; stippled regions, 3′-untranslated sequences. ATG and TGA, translational start and stop codons, respectively. Tet and Amp indicate the genes for tetracyclin and ampicillin resistance. P_{trc} stands for the *trc* promoter present in plasmid pKK233-2.

measuring the release of inorganic phosphate from 3′,5′-cAMP, using the colorimetric assay of Lanzetta et al. (1979). 5′-Nucleotidase and activator-deficient 3′,5′-cyclic nucleotide phosphodiesterase were purchased from Sigma Chemical Co. The assay conditions (final concentrations in 100 μL) were 30 mM Hepes, 30 mM imidazole (pH 7.5), 2 mM $MgCl_2$, 0.1% BSA, 0.02646 unit of 5′-nucleotidase, 0.00184 unit of phosphodiesterase, 1 mM EGTA, $CaCl_2$ as calculated to give the indicated free Ca^{2+} concentration [using a computer program based on Perrin and Sayce (1967) and taking into account 1 mM EGTA/2 mM $MgCl_2$, pH 7.5], and CLP or CaM as indicated. After preincubation of the assay mix for 5 min at 37 °C, the reaction was started by adding cAMP to a final concentration of 1.5 mM. After 10 min at 37 °C, 50 μL of the mixture was transferred to 2 mL of Lanzetta color reagent (Lanzetta et al., 1979), and after 2 h at room temperature, the amount of inorganic phosphate was determined spectrophotometrically at 660 nm.

Ca^{2+}-ATPase Assay. Purified human erythrocyte plasma membrane Ca^{2+}-ATPase, prepared according to Niggli et al. (1979), was a kind gift of Drs. A. Beck-Sickinger and T. Vorherr. The activity of Ca^{2+}-ATPase was measured by the coupled enzyme assay as described (Niggli et al., 1979). The free Ca^{2+} concentration was 10 μM.

RESULTS

Protein Expression. Expression vector pKK-CLP (Figure 1) gives rise to a human CaM-like protein (CLP) with the same number of amino acids (148), but with 23 amino acid substitutions compared to CaM (Figure 2). pKK233-2-derived expression plasmids have been successfully used for the production in *E. coli* of a number of foreign gene products [notably of mammalian CaM (Roberts et al., 1985; West et al., 1988)]. The unusual stability of CaM (and of CLP) against proteolysis, and the surprising nontoxicity of these eukaryotic Ca^{2+}-binding proteins for their host cells, makes them ideal candidates for bacterial overexpression. Accordingly, several milligrams of the purified protein could be obtained from 1 L of bacterial culture. Interestingly, however, the yields of CLP (≤2 mg/L) were consistently lower than those of CaM (≈6 mg/L) (compare also lanes 1 and 2 in Figure 3) although the vector backbone and the sequence surrounding the site of translation initiation are identical for both pKK-CaMIII and pKK-CLP. Amino acid composition and sequence analysis as well as NMR techniques have shown that bacterially expressed mammalian CaM lacks both posttranslational modifications usually associated with this protein, i.e., acetylation of the N-terminus and trimethylation of Lys^{115} (Roberts et al., 1985; Putkey et al., 1985; West et al., 1988). Although it is not yet known whether in vivo expressed CLP carries any posttranslantional modifications at all, it is safe to predict that the protein expressed in *E. coli* will lack any such modifications.

Purification and Characterization. Extracts of bacterial cultures transformed with pKK-CaMIII, pKK-CLP, or, as control, pKK233-2 without any insert were subjected to SDS–polyacrylamide gel electrophoresis (Figure 3, lanes 1–3). Bands could be seen at about 21 kDa for bacterially expressed CaM (Figure 3, lane 1) and at about 17 kDa for CLP (Figure 3, lane 2). The soluble fraction of the bacterial extracts was directly adsorbed in a batch mode to phenyl-Sepharose in the absence of Ca^{2+} to remove unspecific binding proteins. After the supernatant was loaded onto a subsequent phenyl-Sepharose column in the presence of 1 mM $CaCl_2$, the material specifically eluting in a Ca^{2+}-free buffer appeared homogeneous on a SDS–polyacrylamide gel (Figure 3, lanes 4 and 5). Bacterial cultures transformed with pKK233-2 and treated according to the same protocol did not show any corresponding electrophoretic band (Figure 3, lane 6). The successful purification of CLP by Ca^{2+}-dependent phenyl-Sepharose affinity chromatography points to a high structural similarity between CLP and CaM. Despite the 23 amino acid substitutions, CLP is clearly capable of binding Ca^{2+} and of undergoing a conformational change upon Ca^{2+} binding comparable in effect to that displayed by "normal" CaM. The isoelectric points of human CLP and CaM as calculated by the program PEPTIDESORT of the UWGCG software (Devereux et al., 1984) are 4.1 and 3.9, respectively. The higher isoelectric point of CLP than of CaM is easily explained by the significantly higher content of basic residues in the former protein (11 Arg and 7 Lys in CLP, 6 Arg and 8 Lys in CaM; see Figure 2). Bacterially expressed human CaM and CLP were further analyzed by partial amino acid sequencing. Several amino acid differences between CaM and CLP were confirmed as expected from the nucleotide sequence data (e.g., Met^{124}-Ile-Arg-Ala-Ala^{128} in CLP vs Met^{124}-Ile-Arg-Glu-Ala^{128} in CaM). Amino acid analysis of (bacterially expressed) human CaM and human CLP yielded a reasonably good correlation with the expected compositions (Table I). Marked differences between these two proteins are, e.g., apparent in their Arg, Val, and Ile content (Table I and see Figure 2).

Electrophoretic Shift. CaM and other high-affinity Ca^{2+}-binding proteins show a marked Ca^{2+}-dependent change in electrophoretic mobility, even in the presence of SDS (Burgess et al., 1980). This electrophoretic shift is induced by conformational changes in the CaM molecule upon binding of Ca^{2+}. Figure 4 shows the electrophoretic mobility of purified CaM (lanes 1 and 3) and CLP (lanes 2 and 4) in the presence (+Ca^{2+}, lanes 1 and 2) and absence (–Ca^{2+}, lanes 3 and 4) of Ca^{2+}. Both bovine brain CaM (not shown) and bacterially expressed human CaM show the same Ca^{2+}-dependent mobility change (from 21 kDa in the absence to 14 kDa in the presence of Ca^{2+}). Human CLP shifts from an apparent molecular mass of 17.0 kDa in the absence (Figure 4, lane 4) to an apparent molecular mass of 14.5 kDa in the presence (Figure 4, lane 2) of Ca^{2+}. The Ca^{2+}-dependent electrophoretic shift of CLP, albeit smaller than that of CaM, confirms the ability of CLP to undergo Ca^{2+}-dependent conformational changes and its close relationship to CaM.

Calcium Binding. Figure 5 shows a typical Ca^{2+}-binding isotherm in the experimental conditions used for the phosphodiesterase assay (see below). Analysis according to the Adair equation yielded the following stoichiometric binding constants: $K_1 = (3.8 \pm 0.4) \times 10^5\ M^{-1}$, $K_2 = (1.9 \pm 0.2) \times$

```
                       x y z y x  z                               x y z y x  z
      1                                                                           75
CaM   ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARK

CLP   --------VT---------------C---R-----------------R--MS-I-R-----V------G-----

cCM1  -ER-S---------------R----C---M--------------------VG------S---------SL----

SC8   ----------------P---R---Q-V------M--G---------------S----------N----------


                       x y z y x  z                               x y z y x  z
       76                                                                        148
CaM    MKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAK

CLP    -----N-----------------FV----------R-----S---------A--T------------RVLVS-

cCM1   -R-S---------------------------------------------K---CNN----------R---E-

SC8    --G--V---------------**-T----F-----T-----------G------M------I-------V---
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FIGURE 2: Alignment of the amino acid sequence of mammalian CaM (Klee & Vanaman, 1982) with the deduced sequence of CLP (Koller & Strehler, 1988), cCM1 (Stein et al., 1983), and SC8 (Nojima et al., 1987). Identical amino acids are indicated by hyphens, differences by the single-letter code. Deletions are shown by asterisks. x, y, z, y, x, and z refer to amino acid residues acting as ligands in the Ca^{2+} coordination octahedron (Moncrief et al., 1990).

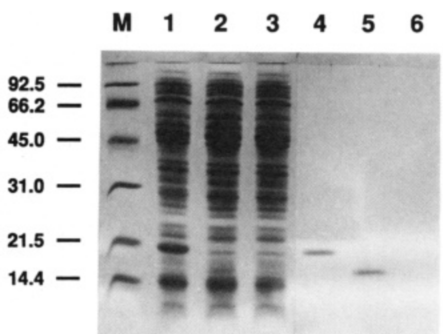

FIGURE 3: SDS–PAGE of crude extracts of *E. coli* JM105 transformed with various expression vectors and of purified CLP and CaM. A 13% SDS–polyacrylamide gel was run with the total protein pellets of 100 μL of *E. coli* cultures carrying pKK-CaM (lane 1), pKK-CLP (lane 2), or pKK233-2 without insert (lane 3). The cultures had been grown in the presence of IPTG for 6 h at 37 °C before aliquots were taken for electrophoresis. Lanes 4 and 5 show purified bacterially expressed human CaM and CLP, respectively, after elution from the phenyl-Sepharose column (1 μg per lane). A control extract (lane 3) treated in the same way yields no visible protein band after phenyl-Sepharose chromatography (lane 6). Protein standards were run on the lane labeled M; their molecular masses in kilodaltons are indicated on the left.

10^5 M^{-1}, $K_3 = (4.9 \pm 1.0) \times 10^4$ M^{-1}, and $K_4 = (1.2 \pm 0.3) \times 10^4$ M^{-1}. The intrinsic constants amount to 1.0×10^5, 1.2×10^5, 7.3×10^4, and 4.8×10^4 M^{-1}, indicating that binding of the first two Ca^{2+} occurs with a somewhat higher affinity than that of the last two Ca^{2+}. The mean affinity constant of 10^5 M^{-1}, determined at the low ionic strength conditions of the PDE assay, is very similar to the one obtained for CaM under conditions of physiological ionic strength. For the sake of comparison, Ca^{2+} binding to CLP was also determined in 50 mM Tris-HCl buffer, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$, and 7.5 mM β-ME. The binding isotherm (data not shown) allowed estimation of the following intrinsic affinity constants: $K'_1 = 3.8 \times 10^3$ M^{-1}, $K'_2 = 1.5 \times 10^4$ M^{-1}, $K'_3 = 7.5 \times 10^3$ M^{-1}, and $K'_4 = 4.0 \times 10^3$ M^{-1}. Thus, the high ionic strength lowers the four constants by a factor of 6.3 (average). A very similar shift of the binding constants was observed in the case of bovine brain calmodulin (Cox et al., 1984). The detailed Ca^{2+}- and Mg^{2+}-binding studies on CLP under physiological ionic strength conditions confirm this general behavior (unpublished results).

Table I: Amino Acid Composition of Bacterially Expressed Human CaM and CLP

amino acid	human CaM[a]	human CLP[a]
Asp	18.1 (17)	16.3 (17)
Glu	21.3 (21)	17.7 (20)
Ser	2.7 (4)	4.8 (6)
Thr	9.1 (12)	7.3 (10)
Gly	11.4 (11)	13.4 (12)
His	1.1 (1)	1.3 (1)
Arg	8.0 (6)	12.9 (11)
Ala	12.5 (11)	9.5 (9)
Pro	2.0 (2)	2.0 (2)
Tyr	1.2 (2)	0.7 (1)
Val	8.8 (7)	11.1 (11)
Ile	8.8 (8)	3.0 (4)
Leu	12.6 (9)	9.7 (10)
Phe	9.3 (8)	6.1 (9)
Lys	8.4 (8)	4.8 (7)

[a] The expected values as calculated from the corresponding amino acid sequences are shown in parentheses.

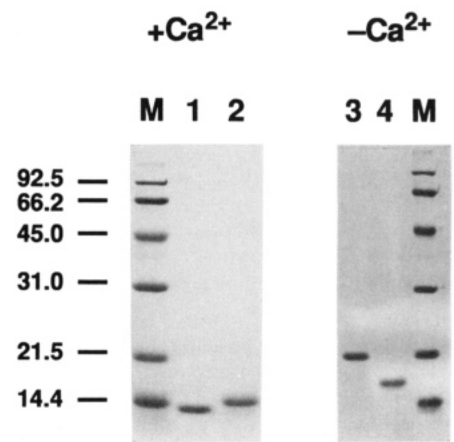

FIGURE 4: Electrophoretic mobility shift of purified CLP and CaM. Purified bacterially expressed human CaM (lanes 1 and 3) and CLP (lanes 2 and 4) were run (1 μg per lane) on a 12% SDS–polyacrylamide gel either in the presence (lanes 1 and 2) or in the absence (lanes 3 and 4) of Ca^{2+} as described under Materials and Methods. M, marker lanes containing protein standards; the molecular masses in kilodaltons are indicated on the left.

Activation of Phosphodiesterase. To obtain information on the functional properties of CLP and to compare them with those of CaM, the activity of bovine heart PDE was measured as a function of the CLP or CaM concentration.

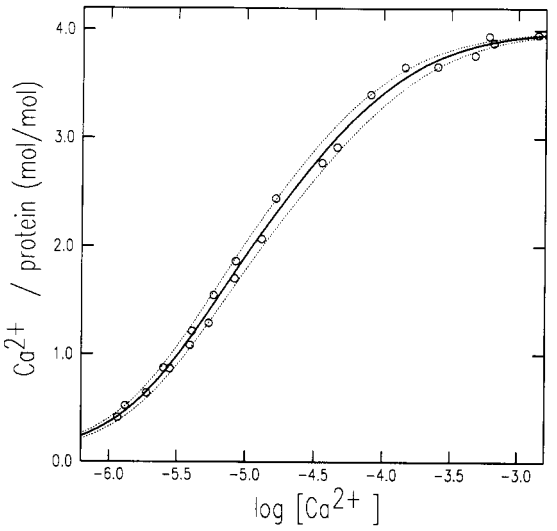

FIGURE 5: Ca^{2+} binding to CLP. Ca^{2+} binding was measured by flow dialysis at 25 °C in the buffer used for PDE assays. The CLP concentration was 70 μM. The solid line represents the isotherm calculated with the Adair equation using the mean stoichiometric constants listed below. The dotted lines delimit the area of uncertainty on each of the constants. The following estimates for the mean constants and their uncertainties were obtained: K_{1Ca} = (3.8 ± 0.4) × 10^5 M^{-1}, K_{2Ca} = (1.9 ± 0.1) × 10^5 M^{-1}, K_{3Ca} = (4.9 ● 1.0) × 10^4 M^{-1}, and K_{4Ca} = (1.2 ± 0.3) × 10^4 M^{-1}.

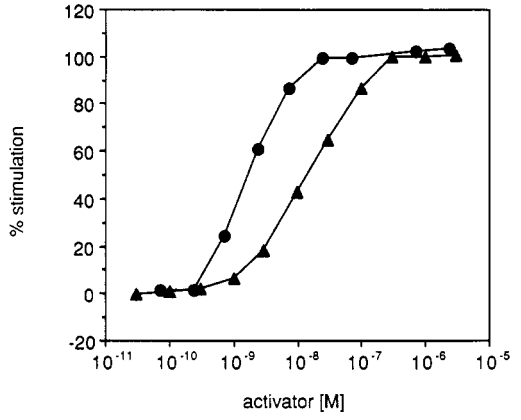

FIGURE 6: Activation of 3′,5′-cyclic nucleotide phosphodiesterase by human CLP. The relative level of PDE stimulation is shown as a function of the concentration of bacterially expressed human CLP filled triangles) and of bacterially expressed human CaM (filled circles). The maximal stimulation of PDE in the presence of CaM is taken as 100%. The free Ca^{2+} concentration was 0.8 mM. Each value represents the mean of three independent measurements.

PDE activation by CLP at a fixed concentration of Ca^{2+} (0.8 mM) is presented in Figure 6 and shows that both bacterially expressed CaM and bacterially expressed CLP were able to stimulate this enzyme to the same maximal extent. No difference was found between bovine brain and bacterially expressed human CaM in their effect on PDE (not shown), confirming previous results (Putkey et al., 1985) that PDE activation is not sensitive to posttranslational modifications of CaM (N-terminal acetylation and trimethylation of Lys^{115}). The K_{act} value for CaM, however, is about 7-fold lower than the K_{act} value for CLP. Obviously, the amino acid substitutions in CLP when compared to CaM do not affect the ability of CLP to maximally stimulate PDE. The affinity of CLP for the PDE target enzyme, however, appears to be reduced. Since at 0.8 mM Ca^{2+} CLP is nearly saturated with Ca^{2+} (see Figure 5), it can be concluded that CLP·Ca_4 activates phosphodiesterase with a K_a of 7.4 × 10^7 M^{-1}. In further experiments, the Ca^{2+} dependence of PDE stimulation by CLP at different

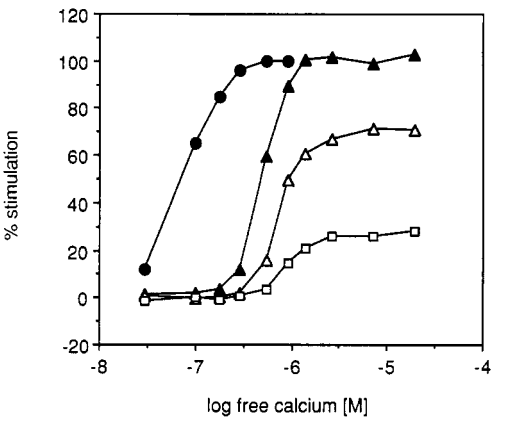

FIGURE 7: [Ca^{2+}] dependence of PDE stimulation by CLP. PDE stimulation (expressed as percent of the maximal value obtained with CaM) is plotted as a function of the Ca^{2+} concentration in the presence of 1 μM bacterially expressed human CaM (filled circles) or 1 μM (filled triangles), 100 nM (open triangles), or 10 nM (open squares) human CLP. Each value represents the mean of three independent measurements.

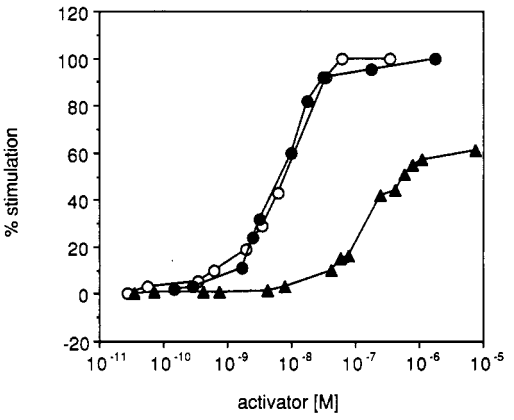

FIGURE 8: Stimulation of plasma membrane Ca^{2+}-ATPase by human CLP. The stimulation of human erythrocyte plasma membrane Ca^{2+}-ATPase is expressed as an increase in activity over the basal ATPase level in the presence of 10 μM free Ca^{2+} (100% increase is the maximal stimulation obtained in the presence of CaM), and as a function of the concentration of human CLP (filled triangles), bacterially expressed human CaM (filled circles), and bovine brain CaM (open circles). Each value represents the mean of three independent determinations.

concentrations was determined (Figure 7). The activation curves are characterized by their steepness and by the fact that half-maximal activation occurs at similar pCa values despite a 100-fold difference in the CLP concentration. A quantitative analysis of the data in Figure 7 in light of the Ca^{2+}-binding properties of CLP was attempted, but no fit to any simple reaction mechanism, such as the one proposed previously for CaM (Cox et al., 1981), was obtained. Either EGTA interferes in this assay or more than one CLP·Ca_n species interacts with PDE with different affinities and/or different amplitudes of activation.

Activation of Ca^{2+}-ATPase. To characterize the activity of CLP on a different CaM target enzyme, plasma membrane Ca^{2+}-ATPase purified from human erythrocytes was selected. Whereas optimal interaction with PDE requires the presence of the N-terminal part of CaM, activation of Ca^{2+}-ATPase is predominantly dependent on the C-terminal part of CaM (Guerini et al., 1984). Because several amino acid residue differences are present between CaM and CLP in their C-terminal halves, different kinetics for Ca^{2+}-ATPase activation by CaM and by CLP might be expected. Figure 8 shows the Ca^{2+}-ATPase activity as a function of CaM or

CLP concentration. Stimulation of the purified Ca^{2+} pump was monitored in the presence of 10 μM free Ca^{2+}, thus guaranteeing Ca^{2+} saturation of the pump and maximal ability to be stimulated by agonists. Bovine brain CaM and bacterially expressed CaM showed indistinguishable pathways of activation with half-maximal values at 6.5 and 6.2 nM, respectively. In marked contrast, CLP behaved differently in stimulating the Ca^{2+}-ATPase, inducing only 62% of the maximal activity obtained with CaM (Figure 8) even at micromolar concentrations. Moreover, the K_{act} value of CLP (110 nM) was about 20-fold higher than the value observed for CaM.

DISCUSSION

CaM has long been thought to exist as a unique and highly conserved Ca^{2+}-binding protein in all higher vertebrates. The identification of chicken (Putkey et al., 1985), rat (Nojima & Sokabe, 1987), and human (Koller & Strehler, 1988) intronless genes containing open reading frames with extensive homology to CaM was therefore an intriguing finding. However, until recently unequivocal proof of the functionality of these "retrogenes" was lacking despite various attempts to detect their expression at the RNA level. Although studies of such CaM "mutants" are interesting per se, the finding of a specific pattern of expression of the human CaM-like gene (Yaswen et al., 1990; Stampfer & Yaswen, 1992) greatly stimulated our interest to study the properties of its product.

Despite the 23 amino acid substitutions of CLP as compared to CaM, CLP binds 4 Ca^{2+} and undergoes Ca^{2+}-dependent conformational changes. A comparison of the amino acid sequence of the known Ca^{2+}-binding sites in CaM with the corresponding sequences in CLP indicates that all loops have retained the ability to bind Ca^{2+} (Figure 2). Except for the $Ala^{57} \rightarrow$ Arg substitution in the second Ca^{2+} loop, substitutions are conservative or have been shown to occur in "natural" CaM molecules of different origin. For example, Cys^{26} in the first loop is also present in plant CaM (Lukas et al., 1984; Toda et al., 1985; Braam & Davis, 1990) and the chicken CaM-like protein cCM1 (Stein et al., 1983). The $Tyr^{99} \rightarrow$ Phe substitution in the third loop is also observed in scallop CaM (Toda et al., 1981), trypanosomal CaM (Tschudi et al., 1985), several fungal CaMs (LéJohn, 1989; Rasmussen et al., 1990), *Chlamydomonas* CaM (Zimmer et al., 1988), and in the known plant CaMs (Lukas et al., 1984; Toda et al., 1985; Braam & Davis, 1990). The α-carbonyl oxygens of Cys^{26} and Phe^{99}, which are the only substituted residues directly involved in binding Ca^{2+}, should still be able to act as ligands in the Ca^{2+} coordination octahedron (Moncrief et al., 1990). Two hydrophobic pockets, formed by the side chains of amino acid residues primarily located in α-helices flanking the Ca^{2+}-binding loops, are exposed in CaM upon binding of Ca^{2+} (Babu et al., 1988). The hydrophobic character of the corresponding regions in CLP has remained unchanged, a fact that helps explain why this protein can be purified on phenyl-Sepharose in a Ca^{2+}-dependent manner. This feature also suggests intact drug-binding sites in CLP and supports its ability to interact with CaM target enzymes.

Modifications (Mann & Vanaman, 1988) and substitutions (Craig et al., 1987) in the central helix of CaM are thought to reduce the affinity of CaM for PDE while still allowing maximal activation. The product of the chicken CaM-like gene cCM1, which contains four substitutions in this region, shows a 3-fold higher K_{act} for activation of PDE than normal CaM (Putkey et al., 1985). In contrast, CLP with only two alterations in this region ($Thr^{70} \rightarrow$ Gly and $Ser^{81} \rightarrow$ Asn) shows an approximately 10-fold increased K_{act} value. As long as the functional integrity of the central helix as a flexible tether is guaranteed (Persechini et al., 1991), the amino acid sequence of this region does not appear to be the main factor determining the association of CaM (or CaM-like proteins) with PDE.

In a recent study using different CaM mutants, charge reversals in the helices located in the carboxy-terminal domain have been shown to strongly affect Ca^{2+}-ATPase activation (Kosk-Kosicka & Bzdega, 1991). The $Asn^{111} \rightarrow$ Arg, $Glu^{127} \rightarrow$ Ala, and $Gln^{143} \rightarrow$ Arg substitutions in the carboxy-terminal half of CLP (1− → 2+) may thus significantly contribute to a lowered affinity of CLP for the Ca^{2+} pump. Five out of six carboxy-terminal amino acids are different in CLP as compared to CaM (Figure 2). Since Ca^{2+}-ATPase activation is sensitive to methionine modifications in the C-terminal half of CaM (Guerini et al., 1987), the two substitutions at positions 144 and 145 of CLP (Met → Val and Met → Leu) might preclude maximal stimulation of the pump.

Recently, Yaswen et al. (1990) reported the existence of mRNA transcribed from the human CLP gene in subpopulations of normal epithelial cells, whereas CLP gene transcripts were undetectable in several tumor-derived cell lines. Using specific antibodies, the same authors also demonstrated the existence and highly cell-type-restricted localization of the corresponding protein (Stampfer & Yaswen, 1992). The specific pattern of expression and its sensitivity toward malignant transformation raise the question of the physiological role of CLP in the cell. Evidence against a universal role for CLP comes from its restricted distribution among cell types and from the absence of a highly homologous protein in other vertebrate species. Notably, the translated products of the chicken CaM-like gene cCM1 (Stein et al., 1983) and of the rat CaM-like gene SC8 (Nojima et al., 1987) show a lower degree of identity to the human CLP (78% and 76%, respectively) than to CaM itself (87%). It therefore seems likely that the CaM-like genes arose independently in chicken, rat, and man. Preliminary experiments using ^{125}I-labeled bacterially expressed CLP as probe to detect specific target proteins on "Western" blots of electrophoretically separated total tissue extracts have so far not allowed the identification of any promising candidate protein (data not shown). Difficulties in the successful application of this method may stem from a reduced affinity of CLP for its target (as appears to be the case for PDE and Ca^{2+}-ATPase) and/or the low abundance of the specific target protein. A different approach that has as yet to be tried in the search for the (presumptive) CLP target consists of identifying proteins specifically retained on "affinity" columns containing immobilized CLP. With bacterially expressed human CLP now at hand in reasonable amounts, it should soon be possible to learn more about the in vivo function of this intriguing protein.

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Registry No. Ca^{2+}, 7440-70-2; ATPase, 9000-83-3; 3′,5′-cyclic nucleotide phosphodiesterase, 9040-59-9.