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Sequence Comparison of the 63-, 61-, and 59-kDa Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterases†

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ABSTRACT: Partial protein sequences from the 59-kDa bovine heart and the 63-kDa bovine brain calmodulin-dependent phosphodiesterases (CaM-PDEs) were determined and compared to the sequence of the 61-kDa isozyme reported by Charbonneau et al. [Charbonneau, H., Kumar, S., Novack, J. P., Blumenthal, D. K., Griffin, P. R., Shabanowitz, J., Hunt, D. F., Beavo, J. A. & Walsh, K. A. (1991) *Biochemistry* (preceding paper in this issue)]. Only a single segment (34 residues) at the N-terminus of the 59-kDa isozyme lacks identity with the 61-kDa isozyme; all other assigned sequence is identical in the two isozymes. Peptides from the 59-kDa isozyme that correspond to residues 23-41 of the 61-kDa protein bind calmodulin with high affinity. The C-terminal halves of these calmodulin-binding peptides are identical to the corresponding 59-kDa sequence; the N-terminal halves differ. The localization of sequence differences within this single segment suggests that the 61- and 59-kDa isozymes are generated from a single gene by tissue-specific alternative RNA splicing. In contrast, partial sequence from the 63-kDa bovine brain CaM-PDE isozyme displays only 67% identity with the 61-kDa isozyme. The differences are dispersed throughout the sequence, suggesting that the 63- and 61-kDa isozymes are encoded by separate but homologous genes.

Cyclic nucleotide phosphodiesterases (PDEs)[1] catalyze the hydrolysis of cAMP and/or cGMP to their corresponding 5′-nucleoside monophosphates. At least five different enzyme families have been identified, and most of these families contain multiple forms of closely related PDEs. One large family, the CaM-dependent PDEs (CaM-PDEs) is particularly critical for intracellular signaling in that they respond to calcium by decreasing the concentration of cAMP and cGMP [for review see Wang et al. (1990)]. Although a number of members of the CaM-PDE family have been described (Beavo, 1988; Beavo & Reifsnyder, 1990), three well-characterized forms are the 59-kDa isozyme isolated from bovine heart and the 61- and 63-kDa isozymes isolated from bovine brain (LaPorte & Storm, 1979; Hansen & Beavo, 1982; Sharma & Wang, 1986, 1987). Sharma and Wang (1986) also described a 58-kDa CaM-PDE from bovine lung that bound

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[1] Abbreviations: PDE, cyclic nucleotide phosphodiesterase; CaM, calcium/calmodulin complex, CaM-PDE, calmodulin-dependent cyclic nucleotide phosphodiesterase; CM, *S*-carboxymethyl; BNPS-skatole, an acronym for the reagent described by Fontana et al. (1973); HPLC, high-performance liquid chromatography; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Pth, phenylthiohydantoin.

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CaM even in the presence of EGTA. Shenolikar et al. (1985) isolated a 75-kDa CaM-stimulated enzyme from bovine brain that is relatively specific for cGMP. More recently, Rossi et al. (1988) have described a murine testis CaM-PDE of 68-kDa that has biphasic kinetics and K_m's for cAMP and cGMP (2 and 20 μM, respectively) that are distinct from those of the 58-, 59-, and 61-kDa isozymes.

Both the 61-kDa brain and 59-kDa heart isozymes are recognized by a monoclonal antibody (ACAP) that binds to the Ca^{2+}/CaM-PDE complex with 100-fold higher affinity than the PDE alone (Hansen & Beavo, 1986). This indicates that these two proteins share epitopes and may undergo similar conformational changes upon binding to Ca^{2+}/CaM. The heart 59-kDa and brain 61-kDa isozymes have nearly identical substrate specificities and kinetic constants. On the basis of peptide mapping experiments, Krinks et al. (1984) suggested that the heart 59-kDa protein could be a proteolytic form of the 61-kDa isozyme. On the other hand, there is evidence of differences among the CaM-PDE isozyme family. For example, the 59- and 61-kDa CaM-PDEs differ in mobility on SDS–PAGE and in elution position on DEAE chromatography. More significantly, the 59-kDa isozyme has at least a 10-fold higher affinity for CaM (Hansen & Beavo, 1986). Oncomodulin (an oncofetal calcium-binding protein) also binds with higher affinity to the heart than to the brain isozyme (Mutus, 1985).

The 63-kDa brain isozyme differs from the 59- and 61-kDa proteins in that it is not recognized by the monoclonal antibody ACAP (Hansen et al., 1986). The 61-kDa isozyme, but not the 63-kDa form, is phosphorylated in vitro by cAMP-dependent protein kinase, while only the 63-kDa isozyme is phosphorylated in vitro by CaM kinase II (Sharma & Wang, 1985; Hashimoto et al., 1989). Despite these differences, the 61- and 63-kDa CaM-PDEs have similar CaM-binding affinities. Peptide maps generated by limited proteolysis with staphylococcal V8 protease (Sharma et al., 1984) suggested that the 61- and 63-kDa bovine brain CaM-PDEs might have different sequences.

In order to understand the structural and functional relationship among the CaM-PDE isozyme family, the complete amino acid sequence of the 61-kDa isozyme (Charbonneau et al., 1990) has been compared with the nearly complete sequence of the 59-kDa CaM-PDE and with partial sequence data from the 63-kDa isozyme. The 61- and 59-kDa sequences differ in a single N-terminal region that is thought to be a site of interaction with CaM. Structural changes at this site may explain the differences in the affinity of these isozymes for CaM. The data also suggest that alternative RNA splicing is responsible for the differences between the 59- and 61-kDa isozymes and that the 63-kDa isozyme is derived from a separate gene.

Experimental Procedures

Enzyme Isolation. The 59-kDa CaM-PDE was purified from 10 kg of fresh bovine ventricle, as previously described (Hansen & Beavo, 1986) with the following modifications. The $MgCl_2$ eluate from the ACAP antibody affinity column was dialyzed against 500 volumes of buffer A (40 mM Tris, pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% (v/v) 2-mercaptoethanol) and applied to a TSK-DEAE HPLC column (Bio-Rad) equilibrated in the same buffer. The CaM-PDE was eluted with a linear gradient of 0–500 mM NaCl in buffer A at a flow rate of 1 mL/min. The activity peak was pooled, diluted 4-fold in buffer A containing 2 mM EGTA instead of calcium, reapplied to the TSK-DEAE column equilibrated in this buffer, and eluted with the same gradient in buffer A containing 2 mM EGTA. CaM-PDE, which initially eluted at 210 mM NaCl, shifted to 90 mM NaCl in the presence of EGTA. The major A_{280}-nm peak, containing 300–600 μg of protein, showed a single 59-kDa band on silver-stained SDS–PAGE.

PDE activity was determined as described previously (Hansen & Beavo, 1986). Protein concentration was determined either by the method of Bradford (1976) with BSA as the standard or by Picotag amino acid analysis (Bidlingmeyer et al., 1984). The two methods gave similar results with the 59- and 63-kDa CaM-PDEs. Isolation of the 63-kDa CaM-PDE was performed according to the method of Hansen et al. (1988). The yield of the enzyme from 2 kg of frozen bovine brain was 120 μg. On SDS–PAGE the purified preparation displayed a single band at an apparent molecular weight of 63 000.

Protein Cleavage. Prior to cleavage, the purified 59- and 63-kDa CaM-PDEs were reduced and alkylated with iodoacetic acid as described (Charbonneau et al., 1991). CNBr cleavage of CM-protein (3–6 nmol) at methionyl residues was performed for 20 h at room temperature in the dark according to the procedure of Gross (1967). CM-CaM-PDE was cleaved at lysyl residues with *Achromobacter* protease I (Masaki et al., 1981) with a 1:300 (w/w) protease:substrate ratio. The digestion was performed in 3 M urea, 40 mM Tris-HCl, pH 9, for 10 h at 37 °C and was terminated by the addition of 10 μL of 9% formic acid. Large fragments were subdigested with trypsin (Cooper Biomedical), *Staphylococcus aureus* V8 protease, (Miles) or at tryptophan with the use of BNPS-skatole as described in the preceding paper (Charbonneau et al., 1991). Mild acid hydrolysis specific for Asp–Pro bonds was performed with 70% formic acid for 24 h (Landos, 1977). Reduced and alkylated 63-kDa CaM-PDE (1 nmol) was cleaved by *Achromobacter* protease I as described above. Yields of the 59-kDa peptides were 0.5–2.5 nmol and of the 63-kDa peptides were 3.0–400 pmol.

Peptide Purification. Peptides obtained from digests of the intact protein were first fractionated by size-exclusion HPLC chromatography on TSK columns (G3000SW or G2000SW) in 6 M guanidine hydrochloride, 10 mM sodium phosphate, pH 6 (Titani, 1986). Pooled fractions containing peptides of similar size were desalted and further purified by reversed-phase HPLC chromatography using 0.1% trifluoroacetic acid/acetonitrile gradients. Peptides of moderate and large size were resolved with the use of Altex Ultrapore RPSC-C3 (4.6 × 75 mm) or Vydac C4 (4.6 × 250 mm) columns. Small peptides were separated with a SynChropak RP-P (C18, 4.1 × 250 mm) column. Reversed-phase HPLC separations were performed with a Varian 5000 liquid chromatograph, monitoring effluent at 206 nm.

Automated Sequence Analysis and Amino Acid Analysis. Peptides available in quantities greater than 1 nmol were sequenced with a Beckman Model C spinning-cup sequencer as described (Charbonneau et al., 1985). With less than 1 nmol of peptide, an Applied Biosystems Model 470A gas-phase sequencer was used with programs adapted from those provided by the manufacturer. Pth-amino acids were identified by reversed-phase HPLC with an IBM Cyano column as described by Hunkapiller and Hood (1983). In many cases, Pth-amino acid identifications were confirmed on a second HPLC system (Ericsson et al., 1977) with a Du Pont Zorbax ODS column. During the later phases of this study the IBM Cyano column was replaced with a Du Pont Zorbax PTH column (eluted isocratically with a phosphate tetrahydrofuran/acetonitrile buffer at pH 3 according to the manufac-

FIGURE 1: Proof of sequence for six large segments of the 59-kDa CaM-PDE. The underlined sequences summarize segments of overlapped peptides from the 59-kDa CaM-PDE. Peptide sequences deduced from Edman degradation are listed below the underline. Hyphens denote unidentified sequences, and arrowheads represent peptides for which the sequence may continue beyond the last identified residue. Individual peptides were generated by cleavage of the 59-kDa protein at methionyl or lysyl residues. These larger peptides were then subdigested with trypsin (at lysyl and arginyl residues), with staphylococcal V8 protease (at glutamyl residues), with BNPS-skatole (tryptophan residues), or by limited acid cleavage (see Experimental Procedures section).

turers' instructions), and an Applied Biosystems Model 120A on-line Pth analyzer was installed on the gas-phase sequencer. For some analyses, a Model 477A liquid pulse sequencer (Applied Biosystems) with a Model 120A on-line Pth analyzer was used as described above. Amino acid analyses were obtained by the reversed-phase separation of the phenyl thiocarbamoyl derivatives on a Waters Picotag system as described by Bidlingmeyer et al. (1984).

Synthetic Peptide Inhibition of CaM-PDE Activity. Peptide analogues were synthesized by solid-phase procedures on an Applied Biosystems Model 430A peptide synthesizer. Peptides P59C16 and P59C21 were synthesized with use of the t-Boc method (Barany & Merrifield, 1979), whereas the F-moc procedure was used for the P63C15 peptide analogue. P59C21 was synthesized as the carboxyl-terminal amide derivative. Peptides were purified by preparative HPLC chromatography, and their structures were verified by gas-phase sequencing. The peptide P63C15 was dissolved and stored in solutions containing 2-mercaptoethanol in order to prevent oxidation of the methionine residues. The ability of peptides to inhibit calmodulin-dependent activation of the 61-kDa CaM-PDE was examined by use of assay procedures described in the preceding paper (Charbonneau et al., 1991). Direct binding interactions between the peptides and CaM were demonstrated by CaM-Sepharose chromatography as described in the preceding paper (Charbonneau et al., 1991).

RESULTS

Partial sequence data were obtained from the analysis of peptides generated by the cleavage of the reduced and alkylated 59- and 63-kDa CaM-PDEs at lysyl residues. The 59-kDa protein was also cleaved with CNBr at methionyl residues to generate peptides overlapping the lysyl peptides. Additional overlaps and internal sequence data were generated by subdigesting large methionyl or lysyl fragments of the 59-kDa protein with trypsin, staphylococcal V8 protease, or selective Asp-Pro cleavage as described in the Experimental Procedures section.

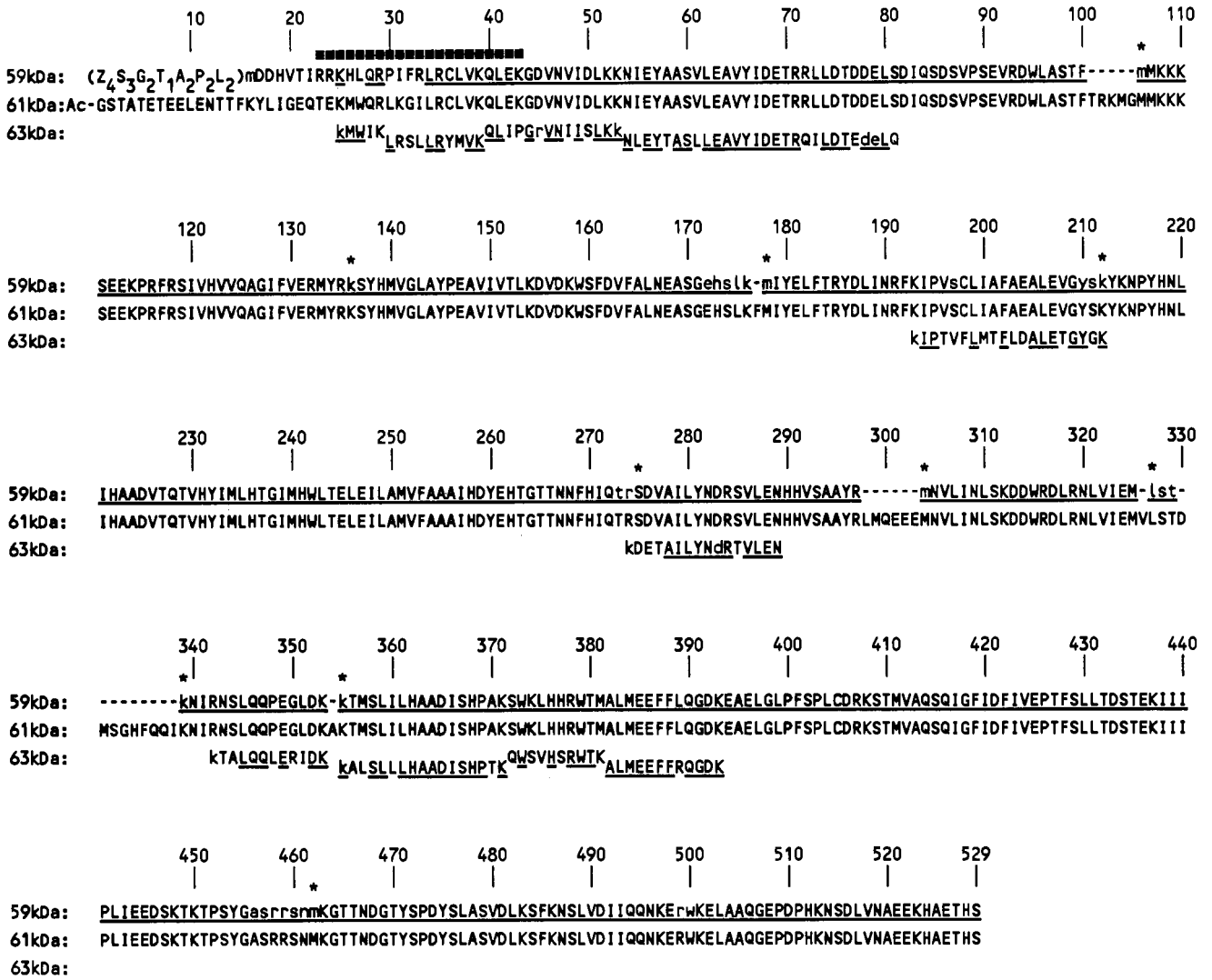

FIGURE 2: Sequences from the 59- (heart) and 63- (brain) kDa CaM-PDEs aligned with the complete sequence of the 61-kDa isozyme (Charbonneau et al., 1991). Residue numbers corresponding to the 61-kDa isozyme are underlined. Tentative identifications are in lower case letters, and hyphens denote unidentified residues. The N-terminus of the 59-kDa isozyme, as determined by the subtraction of a methionyl peptide (mDDHVTIRRK) from the composition of an amino-terminal-blocked lysyl peptide, is in parentheses. Asterisks denote positions where segments of the 59-kDa sequence have not been overlapped. Solid boxes are placed above residues within the CaM-binding sites identified in the 61-kDa isozyme (Charbonneau et al., 1991).

The sequences of six large segments (I–VI in Figure 1) were deduced from the analysis of peptides from the 59-kDa isozyme. These six segments plus five nonoverlapped peptides placed 491 residues as summarized in Table I and Figure 1. Cleavage of the 63-kDa isozyme at lysyl residues yielded 13 pure peptides that were subjected to sequence analysis. The results identify 13 nonoverlapping sequences totaling 187 residues (Table II).

Comparison of the 59- and 61-kDa Isozymes. Elution profiles from size-exclusion HPLC chromatography of the *Achromobacter* protease I digests (not shown) suggested that the 59- and 61-kDa proteins might be very similar, whereas that of the 63-kDa enzyme was significantly different. As the sequence of the 59-kDa protein emerged, it was evident from a comparison with the 61-kDa sequence that, except for their N-terminal regions, the two proteins were identical at all positions where sequence was available. The six large segments (Figure 1) and all five peptides (Table I) determined for the 59-kDa isozyme were readily aligned with corresponding sequences of the 61-kDa isozyme as illustrated in Figure 2. Of the 529 residues of the 61-kDa isozyme, 491 were aligned with sequences from the 59-kDa isozyme, and all available 59-kDa sequences that were aligned between residues 34 and 529 of the 61-kDa protein[2] were identical. As shown in the alignment of Figure 2, there are ten positions where fragments from the 59-kDa protein are not overlapped. Given that the two sequences are nearly identical, it is likely that the 59-kDa fragments have been placed in the proper order by alignment to the 61-kDa sequence; however, the presence of additional residues at the site of the missing overlaps cannot be excluded. There are six gaps (ranging from one to nine residues in length) where no corresponding residues for the 59-kDa enzyme have been identified. The missing residues appear to result either from the failure to isolate the corresponding peptide or from the inability to extend the sequences of some peptides to their C-termini. However, the possibility that these residues are deleted from the 59-kDa enzyme cannot be eliminated on the basis of these data.

The C-terminus of the 59-kDa enzyme was determined from a 19-residue fragment isolated after acid cleavage of the whole protein at an Asp–Pro bond. This peptide ended with the sequence ETHS (in segment VI, Figure 1), which corresponds

[2] Residue numbers given for the 59- and 63-kDa isozymes refer to corresponding positions in the 61-kDa CaM-PDE sequence described in the preceding paper (Charbonneau et al., 1991).

Table I: Partial Sequence Data from the 59-kDa CaM-PDE Isozyme

residue no. in 61-kDa CaM-PDE	corresponding sequence observed in 59-kDa CaM-PDE: peptide source[a]	no. of residues	no. of residues not identified
1–15	composition only		15[b]
16–100	segment I	85	
101–105	ND[c]		5
106–136	segment II	31	
137–176	segment III	40	
177	ND		1
178–211	M4	34	
212–274	segment IV	63	
275–284	M5-T1	10	
285–297	M5-T2	13	
298–303	ND		6
304–329	M6	25	1
330–338	ND		9
339–352	K9	14	
353	ND		1
354–461	segment V	108	
462–529	segment VI	68	
total		491	41

[a] Segments of overlapping peptides (I–VI) are illustrated in Figure 1. M4 and M6 are products of cleavage with CNBr. K9 is a product of cleavage at lysine. M5–T1 and M5–T2 are tryptic subdigestion products of the CNBr fragment M5. [b] Amino acid analyses indicated that 16 residues in the 59-kDa isozyme replace the N-terminal 15 residues of the 61-kDa isozyme. [c] ND indicates that these sequences were not determined.

to the C-terminus of its 61-kDa counterpart. This was supported by the amino acid composition of a cyanogen bromide peptide lacking homoserine (data not shown).

Like the 61-kDa isozyme, the amino terminus of the 59-kDa isozyme was blocked. However, the following data show that the sequence of the amino-terminal 33 residues of the 61-kDa isozyme is different from that of the 59-kDa isozyme. An 85-residue segment of the 59-kDa protein (segment I in Figure 1 and Table I) displays identity of its C-terminal 67 residues with residues 34–100 of the 61-kDa enzyme, but its N-terminal 18-residue sequence, MDDHVTIRRKHLQRPIFR, was quite different from the corresponding sequence, KYLIGEQTEKMWQRLKGI, in the 61-kDa enzyme (residues 16–33, Figure 2). Attempts to extend the analysis of the 59-kDa enzyme toward the N-terminus were not successful with use of three separate preparations of a blocked peptide isolated after lysyl cleavage. This peptide was resistant to subdigestion, and attempts to analyze it by mass spectrometry were unsuccessful. In our hands, this peptide appeared to adsorb nonspecifically to surfaces, making it difficult to recover

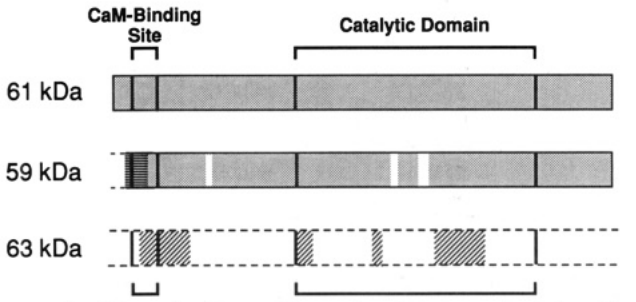

FIGURE 3: Schematic diagram illustrating the structural relationship among the 59-, 61-, and 63-kDa CaM-PDEs. Open areas represent unknown sequence, whereas shaded areas represent regions of known sequence. Segments with identical sequences are shaded with the same pattern. The position of the conserved catalytic domain and a segment with calmodulin-binding properties are indicated.

quantities sufficient for additional manipulations or analyses. However, amino acid compositions were obtained to provide limited characterization. These compositions, after subtraction of the proven sequence of residues 16–25, indicate that 16 residues are yet to be placed at the N-terminus of the 59-kDa enzyme (Figure 2, in parentheses). According to the composition, the sequence of these residues is different from the corresponding 15-residue amino-terminal segment of the 61-kDa protein.

Comparison of the 61- and 63-kDa Isozyme. As shown in Table II, 10 of the 13 lysyl peptides from the 63-kDa protein can be aligned with the 61-kDa sequence with greater than 50% sequence identity. Three peptides from the 63-kDa digest could not be unambiguously aligned by visual inspection or computer-assisted searches. Hence, 138 residues identified from the 63-kDa isozyme could be aligned in four noncontiguous segments (Table II, Figure 2). This similarity is sufficient to suggest a homologous relationship between the 63- and 61-kDa enzymes that is distinct from that of the long segments of identity that characterize the 59/61-kDa comparison. The relationships among these three isozymes is illustrated schematically in Figure 3.

Evidence for the Location of the Calmodulin-Binding Site within the 59- and 63-kDa Isozymes. A major goal in examining the primary sequences of the 59- and 63-kDa CaM-PDE isozymes was to identify and compare their calmodulin-binding sites. In the preceding paper, Charbonneau et al. (1991) have shown that residues 23–44 of the 61-kDa enzyme are involved in binding calmodulin. It is of particular interest that this region spans both identical and nonidentical segments of the 59-kDa isozyme. Since the affinity of the two isozymes for calmodulin differs 20-fold (Hansen & Beavo, 1986), the N-terminal sequence changes may account for the difference

Table II: Sequence of the Lysyl Peptides Derived by *Achromobacter* Protease I Digestion of the 63-kDa CaM-PDE

63-kDa peptides	residue No.[b]	no. of residues	no. identical	% sequence identity
MWIK	26–29	4	2	50
LRSLLRYMVK	30–39	10	5	50
QLIPGrVNIISLKk[a]	40–53	14	9	64
NLEYTASLLEAVYIDETRQILDTEdeLQ	54–81	28	21	75
IPTVFLMTFLDALETGYGK	194–212	18	10	56
DETAILYNdRTVLEN	275–289	15	11	73
TALQQLERIDK	343–353	11	6	55
ALSLLLHAADISHPTK	356–371	16	12	75
QWSVHSRWTK	372–381	10	5	50
ALMEEFFRQGDK	382–393	12	11	92
totals[c]		138	92	67

[a] Upper and lower case residues represent identified and tentative residues, respectively. Hyphens represent unidentified residues. [b] Residue numbers given are those of the 61-kDa isozyme (Charbonneau et al., 1991). [c] Three additional peptides, present in relatively small amounts, could not be aligned with the 61-kDa isozyme; one of these was derived from the β-chain of S-100.

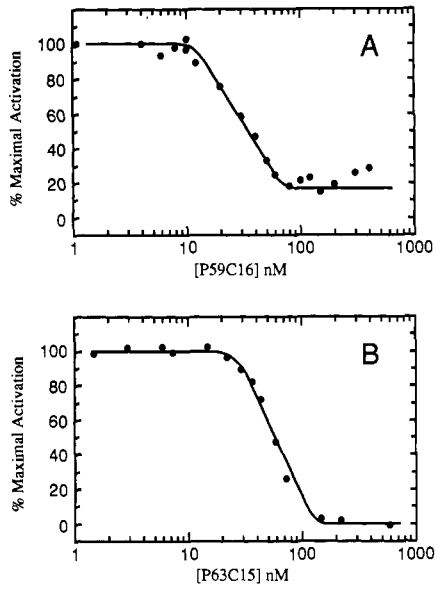

FIGURE 4: Inhibition of the calmodulin-dependent activation of the 61-kDa CaM-PDE by synthetic peptide analogues: (A) inhibition by P59C16 (VTIRRKHLQRPIFRLRCLVKQL), an analogue of the 59-kDa CaM-PDE; (B) inhibition by P63C15 (KMWIKLRSLLRYMVK), an analogue of the 63-kDa CaM-PDE sequence. Activity of a partially activated 61-kDa CaM-PDE preparation was measured by the colorimetric method described by Charbonneau et al. (1991). Assay buffers included 250 μM calcium, 2.7 nM CaM (subsaturating), 0.3 nM PDE, and the indicated concentrations of peptide. Percent maximal activity is defined as $(R_o - R_e/R_m - R_e) \times 100$, where R_e is the basal rate in the presence of EGTA, R_m is the rate without added peptide, and R_o is the rate observed with added peptide. Each point is the average of triplicate activity measurements. Peptide concentration was determined by amino acid composition. The curves were drawn by simple visual inspection of the data.

in affinity. P59C16, a peptide analogue (VTIRRKHLQRPTFRLRCLVKQL) corresponding to residues 20–41 of the 59-kDa isozyme (Figure 2), was found to prevent calmodulin-dependent activation of the 61-kDa isozyme with an IC_{50} of about 30 nM (Figure 4A). A more C-terminal overlapping synthetic peptide, P59C21 (RRKHLQRPIFRLRCLVKQLEK-amide; residues 23–43), gave nearly identical results in a similar experiment (data not shown). In controls, the peptides had no effect on basal activity measured in the presence of 2 mM EGTA and calmodulin added in excess of the peptides overcame the inhibition. These results suggest that the peptides interact directly with calmodulin.

Direct calcium-dependent binding of the peptide P59C16 to CaM was demonstrated with use of CaM–Sepharose. When 15 nmol of this peptide was applied to a CaM–Sepharose column (1.0 mg CaM/mL; 0.7 × 1.7 cm) in buffer containing 100 μM $CaCl_2$, 96% of the peptide was bound and about 50% was recovered by elution with 2 mM EGTA. Both the inhibition data and the CaM–Sepharose binding data indicate that residues 23–41 of the 59-kDa CaM-PDE can bind CaM with high affinity in a calcium-dependent manner.

Sequences from the 63-kDa isozyme can also be aligned to the proposed CaM-binding site (Charbonneau et al., 1991) of the 61-kDa isozyme (Figure 2). Since the 63-kDa sequences from this region are similar and also have a net positive charge and amphiphilic character, a peptide analogue (P63C15) corresponding to residues 25–39 (KMWIKLRSLLRYMVK) was synthesized and tested for its ability to inhibit the calmodulin-dependent activation of the 61-kDa CaM-PDE. As shown in Figure 4B, P63C15 inhibits activation over a concentration range similar to that of the 59-kDa peptide analogue (Figure 4A). The inhibitory effects of P63C15 were reversed by addition of excess calmodulin, and there was no effect of peptide on the basal activity of the PDE (data not shown). These results indicate that the peptide binds directly to calmodulin with high affinity.

Discussion

The results of sequence analysis of the 59- and 63-kDa isozymes of bovine CaM-dependent PDE demonstrate that both sequences are related to the 61-kDa isozyme, but in different ways. All available 59-kDa isozyme sequences are identical with the those of 61-kDa isozyme except for those within a short N-terminal segment. In contrast, more limited data from the 63-kDa form indicate that it may differ by more than 30% and that those differences are distributed throughout its length (Figures 2 and 3). Krinks et al. (1984) proposed, on the basis of comparative peptide maps, that the 59-kDa isozyme might be a product of limited proteolysis of the 61-kDa brain isozyme; however, N-terminal sequence differences indicate that this is not the case (Figure 3). The relationship between the 59- and 61-kDa isozymes is most consistent with their generation from a single gene by tissue-specific, alternative RNA splicing, but the existence of separate genes can not be excluded. The 63/61-kDa relationship suggests that the two isozymes are encoded by separate genes, implying that these two proteins evolved by gene duplication and divergent evolution. Neither the 59-kDa nor the 63-kDa sequences have been completely determined, but the available evidence (92% of 59 kDa; 26% of 63 kDa) supports these hypotheses of the origin of their similarities.

Although only 26% of the 63-kDa brain isozyme sequence has been determined, 10 segments of these sequences align with four regions of both the 61- and 59-kDa isozymes between residues 25 and 393 (Figures 2 and 3). If this pattern of relatedness holds elsewhere in the 63-kDa isozyme, a homologous relationship will be established that contrasts markedly with the virtual identity of the 59-kDa/61-kDa pair. Sharma et al. (1984) proposed, on the basis of peptide maps, that the 63- and 61-kDa proteins were different. The present data confirm this observation and demonstrate that the differences are spread throughout the proteins. A recent report (Grewal, 1989) suggests that the brain 63-kDa isozyme has a much greater cGMP selectivity than the 61-kDa brain isozyme. Presumably, structural differences in their active sites underlie this difference and complete sequence comparisons, when available, may reveal structural features involved in this nucleotide selectivity.

Charbonneau et al. (1986, 1991) have shown that the catalytic domain common to this family of phosphodiesterases corresponds to residues 193–446 of the 61-kDa isozyme. A total of 93% of these residues has been observed in the 59-kDa isozyme, and the sequences are identical. It is likely that the two catalytic domains are identical. However, the two enzymes have been reported to exhibit 10–20-fold differences in responsiveness to Ca^{2+}/CaM (Hansen & Beavo, 1986), and it is of interest to examine the small N-terminal segments of differing structure for the basis of this difference in sensitivity to regulator. Significantly, the only proven difference in sequence (residues 1–33 in the 61-kDa enzyme) overlaps a segment (residues 23–44 in the 61-kDa enzyme) that encompasses a calmodulin-binding site (see preceding paper; Charbonneau et al., 1991).

Although no single consensus sequence has been reported for interaction with calmodulin, the sequences of most calmodulin-binding domains contain both basic and hydrophobic residues, which are predicted to form an amphiphilic helix

(Degrado et al., 1987). Residues 23–44 in the 61-kDa enzyme and the corresponding residues in the 59-kDa isozyme both have these properties. As shown in Figure 4A, a peptide analogue corresponding to the positive, amphiphilic region of the 59-kDa isozyme (residues 20–41 of the 61-kDa isozyme; Figure 2) inhibits the activation of CaM-PDE with an IC_{50} of ≈30 nM, supporting the contention that this region of the 59-kDa protein may form part of a CaM-binding site. The 59- and 61-kDa peptide (Charbonneau et al., 1991) analogues appear to have similar affinities for calmodulin and, by themselves, do not account for the 10–20-fold difference in affinity for calmodulin reported for the native proteins. This suggests that the peptides may not include all residues comprising the calmodulin-binding site; for instance, the unidentified residues at the N-terminus may also be involved in calmodulin binding. Alternatively, the conformation necessary for maximal affinity may not be a favored solution structure for the 22-residue peptide analogue. Clearly, more data are needed to provide specific binding constants and to test hypotheses relating the differences in regulatory response to specific structural alterations in this region.

Sequences from the 63-kDa isozyme were aligned with a portion of the CaM-binding site from the 61-kDa isozyme (residues 25–39 in Figure 2). The peptide analogue P63C15 inhibits the calmodulin-dependent activation of CaM-PDE at nanomolar concentrations (Figure 4B), suggesting that this region of the 63-kDa PDE may form a part of its calmodulin-binding site. Taken together, these studies suggest that the residues comprising the major calmodulin recognition sites within each of the three isozymes have distinct but closely related sequences. With the 63- and 61-kDa sites, sequence differences are scattered over the region; in contrast, differences between the 59- and 61-kDa sites are localized to the N-terminal half of the site. Of the three segments, the 63- and 61-kDa isozymes are the most similar. This may reflect the similarity in CaM affinity between the 63- and 61-kDa isozymes (Sharma & Wang, 1984).

Our findings indicate that the diversity among these three CaM-PDEs reflects differences in primary structure and is not due to proteolytic processing, either physiological or artifactual. The structural data presented provide evidence that the 61- and 63-kDa isozymes are encoded by two distinct genes. The 59/61-kDa CaM-PDE relationship, however, is consistent with the possibility that the observed sequence difference results from alternative RNA splicing of a single gene (Novack et al., 1988). With these two isozymes, differential exon usage could provide a mechanism for generating PDEs with identical kinetic parameters and specificity for cyclic nucleotides but different affinities for CaM. Thus, alternative RNA processing may generate CaM-PDEs that are adapted to respond differently to the calcium/CaM signals in various cell types or intracellular compartments. Davis et al. (1989) have suggested that alternative splicing may generate diversity among rat brain homologues of the *Drosophila* dunce PDE. Alternative splicing has also been shown (Podgorski et al., 1989) for a PDE gene in Dictyostelium; however, the enzyme encoded by this gene is not structurally related to the mammalian PDEs.

The relationship of other proteins in the CaM-PDE family to these three isozymes remains to be elucidated. For example, the kinetic parameters and monoclonal antibody reactivity of the 58-kDa CaM-PDE from bovine lung are similar to the 59- and 61-kDa isozymes, but the lung enzyme appears to have calmodulin bound as an integral subunit (Sharma & Wang, 1986). At present, no structural data are available, but it is possible that the lung enzyme is produced by alternative splicing of a third exon of the 59/61-kDa gene. The structural data presented here should facilitate the design of isozyme-specific antibodies and inhibitors that can be used to investigate the structural relationship among proteins of the CaM-PDE family, as well as their cellular distribution and physiological roles.

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Regulation of c-*jun* Gene Expression in HL-60 Leukemia Cells by 1-β-D-Arabinofuranosylcytosine. Potential Involvement of a Protein Kinase C Dependent Mechanism†

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ABSTRACT: 1-β-D-Arabinofuranosylcytosine (ara-C) is an effective chemotherapeutic agent that incorporates into DNA and results in DNA fragmentation. Recent work has demonstrated that ara-C transiently induces expression of the c-*jun* immediate early response gene. The present studies in HL-60 myeloid leukemia cells extend these findings by demonstrating that the increase in c-*jun* mRNA levels at 6 h of ara-C treatment is regulated by a transcriptional mechanism. In contrast, the subsequent down-regulation of c-*jun* expression is controlled by a posttranscriptional decrease in the stability of the c-*jun* transcripts. Previous work in phorbol ester treated cells has indicated that c-*jun* expression is regulated by the activation of protein kinase C. The present results demonstrate that protein kinase C activity is increased in ara-C-treated cells. This increase was maximal at 60 min and remained detectable through 6 h of ara-C exposure. Moreover, the induction of c-*jun* transcripts by ara-C was inhibited by the isoquinolinesulfonamide derivative H7, but not by HA1004, suggesting that this effect is mediated by protein kinase C. Ara-C-induced c-*jun* expression was also inhibited by staurosporine, another inhibitor of protein kinase C. Taken together, these results indicate that the cellular response to ara-C includes the activation of protein kinase C and that ara-C potentially induces c-*jun* transcription by a protein kinase C dependent signaling mechanism.

The compound 1-β-D-arabinofuranosylcytosine (ara-C)[1] is one of the most effective agents in the treatment of acute myelogenous leukemia (Frei et al., 1969). Ara-C incorporates into leukemic cell DNA (Kufe et al., 1980; Major et al., 1981). The extent of (ara-C)DNA formation correlates with the inhibition of DNA synthesis and the loss of clonogenic survival (Kufe et al., 1980; Major et al., 1981, 1982; Kufe et al., 1984). More recent studies have demonstrated that the inhibitory effects of ara-C are related to both incorporation into DNA and sequence of the DNA template (Townsend & Cheng, 1987: Ohno et al., 1988). Moreover, the inhibition of DNA synthesis by ara-C is associated with DNA fragmentation and endonucleolytic cleavage (Fram & Kufe, 1982; Gunji et al., 1991). These findings are in concert with the conformational and hydrogen-bonding differences of the arabinose sugar moiety altering reactivity of the 3′ terminus and slowing DNA chain elongation (Sundaralingam, 1975; Cozzarelli, 1977). The precise mechanism(s) of action of ara-C and the basis for selectivity against leukemic cells, however, remain unclear.

The c-*jun* protooncogene has been implicated in the regulation of cellular growth and differentiation. This gene is induced as an immediate early event following treatment of fibroblasts with serum, growth factors, and phorbol esters (Ryder & Nathans, 1988; Quantin & Breathnach, 1988; Brenner et al., 1989; Wu et al., 1989; Pertovaara et al., 1989; Ryseck, et al., 1988). The c-*jun* gene codes for the major form of the transcription factor AP-1 (Bohmann et al., 1987; Angel et al., 1987, 1988a; Chiu et al., 1988; Lee et al., 1987). Jun homodimers bind to a heptameric DNA consensus sequence $TGA^{G}/_{C}TCA$ (TRE) that regulates the transcription of genes responsive to growth factors and phorbol esters (Angel et al., 1987, 1986; Lee et al., 1987; Chiu et al., 1987). The DNA binding affinity of Jun/Ap-1 is modulated by the formation of complexes with other factors that contain a leucine zipper and a region rich in basic amino acids (Chiu et al., 1988; Kouzarides & Ziff, 1988; Halazonetis et al., 1988; Rauscher

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[1] Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; TRE, phorbol ester responsive element; CHX, cycloheximide; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; STSP, staurosporine.

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