Alternative Splicing Introduces a Nuclear Localization Signal That Targets Multifunctional CaM Kinase to the Nucleus

Mallika Srinivasan, Carl F. Edman, and Howard Schulman

Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305-5401

Abstract. Intracellular targeting may enable protein kinases with broad substrate-specificities, such as multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) to achieve a selectivity of action in vivo. We have examined the intracellular targeting of three δ-CaM kinase isoforms. The δ_B-CaM kinase isoform is targeted to the nucleus in transfected cells while the δ_A- and δ_C-CaM kinase isoforms are cytosolic/cytoskeletal. A chimeric construct of α-CaM kinase containing the δ_B-CaM kinase variable domain is rerouted to the nucleus while the native α-CaM kinase and chimeras of α-CaM kinase which contain the δ_A- or δ_C-CaM kinase variable domains are retained in the cytoplasm. Using site-directed mutagenesis, we have defined a nuclear localization signal (NLS) within an 11-amino acid sequence, likely inserted by alternative splicing, in the variable domain of δ_B-CaM kinase. Isoform-specific nuclear targeting of CaM kinase is probably a key mechanism in the selective regulation of nuclear functions by CaM kinase.

CaM kinase is a multimer that can be composed of several isoforms. We find that when cells express two different isoforms of CaM kinase, cellular targeting is determined by the ratio of the isoforms. When an excess of the cytoplasmic isoform of CaM kinase is coexpressed along with the nuclear isoform, both isoforms are localized in the cytoplasm. Conversely an excess of the nuclear isoform can reroute the cytoplasmic isoform to the nucleus. The nuclear isoform likely coassembles with the cytosolic isoform, to form a heteromultimeric holoenzyme which is transported into the nucleus. These experiments demonstrate isoform-specific targeting of CaM kinase and indicate that such targeting can be modified by the expression of multiple isoforms of the enzyme.

GROWTH factors, neurotransmitters and hormones typically modify intracellular processes throughout the cell with high specificity and speed. Their action is often mediated by protein kinases with broad substrate specificities that enable them to coordinate multiple responses. However, the ability of such kinases to phosphorylate many proteins, including non-physiological substrates in vitro, suggests that there must be mechanisms for refining their target specificity in situ. We have examined this issue by studying multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase or CaM kinase II),[1] a major intracellular mediator of Ca^{2+} action (for review see Hanson and Schulman, 1992*b*). Our working model is a modification of the targeting subunit hypothesis (Hubbard and Cohen, 1993). Stated simply, the hypothesis suggests that a targeting domain (or subunit) directs the catalytic domain (or subunit) of the kinase to the substrate locus. Positioning of the kinase near its substrate or target may occur via a nearby anchoring protein which interacts with the targeting domain or by direct binding of this domain with the substrate. Such targeting, along with spatial/temporal control of the Ca^{2+} signal, may underlie target specificity of signal transduction via CaM kinase.

Some of nature's solutions for specificity have been provided by the study of other kinases and phosphatases. Protein phosphatase 1 is targeted to glycogen or myofibrils by virtue of a targeting subunit which associates with its catalytic subunit (Hubbard and Cohen, 1993). The type II cAMP-dependent protein kinase (PKA) is targeted via its regulatory subunit to binding proteins on microtubules or postsynaptic density (Scott and Soderling, 1992). Protein kinase C has been shown to translocate to receptor proteins that anchor it near its substrates (Smith and Mochly-Rosen, 1992). Such targeting helps to specify which of the many substrates of the kinase/phosphatase would be phosphorylated/dephosphorylated and to increase the efficiency of the reaction by increasing the local concentration of the kinase/

Address correspondence to Dr. Howard Schulman, Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305-5401. Telephone: (415) 723-7668; FAX: (415) 725-3958.

The current address of Dr. Carl F. Edman is Cancer Center, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0636.

1. *Abbreviations used in this paper*: CaM kinase, multifunctional Ca^{2+}/calmodulin-dependent protein kinase; PKA, cAMP-dependent protein kinase; Ag, antigen; (H), Haemagglutinin peptide-tag; NLS, nuclear localization signal.

phosphatase. Distinct patterns of phosphorylation could be achieved in different cell types by the selective expression of a kinase/phosphatase isoform, intracellular anchoring protein, and substrates.

CaM kinase is distributed in a wide variety of cell types where it is found localized in the cytosol, cytoskeleton, membrane, and nucleus. Its broad specificity and distribution enables it to phosphorylate substrate proteins localized to the cytosol, cytoskeleton, membrane, and nucleus. It can thus regulate contractility, gene expression, synthesis and release of neurotransmitters, and ion permeation across cell membranes. For example, CaM kinase phosphorylates microtubule-associated protein 2 (MAP-2) and tau on microtubules, phospholamban in sarcoplasmic reticulum, synapsin I on synaptic vesicles, and tyrosine hydroxylase and pyruvate kinase in the cytosol (for review see Hanson and Schulman, 1992*b*).

How does CaM kinase participate in regulatory processes in the nucleus? A nuclear localization of a presumably nonneuronal form(s) of CaM kinase has been demonstrated by immunocytochemistry in fibroblasts and glioma cells (Ohta et al., 1990). Nuclear forms of CaM kinase may be responsible for the increasing number of nuclear functions being attributed to CaM kinase. It regulates nuclear envelope breakdown in sea urchin eggs (Baitinger et al., 1990), releases Xenopus oocytes from meiotic metaphase arrest (Lorca et al., 1993) and promotes maturation of oocytes (Waldmann et al., 1990), regulates G_2-M transition in mammalian cells (Planas-Silva and Means, 1992), and induces gene expression via the phosphorylation of the CCAAT enhancer element-binding protein β (C/EBPβ) (Wegner et al., 1992). Whereas activation of PKA frees its catalytic subunit so that it is no longer excluded from the nucleus (Nigg et al., 1985), there is no evidence to suggest that CaM kinase dissociates into monomers after activation. The large size of the kinase (a decameric protein of 500–600 kD) makes it unlikely to mediate nuclear events without specific targeting into the nucleus. In the absence of an identified nuclear isoform of multifunctional CaM kinase, some researchers have begun to examine other CaM kinases such as CaM kinase I and CaM kinase IV which are monomeric and less likely to be excluded from the nucleus.

Multifunctional CaM kinase is composed of a family of isoforms derived from four (α, β, γ and δ) closely related genes (for review see Rostas and Dunkley, 1992). Isoforms range in size from 54 to 60 kD and form multimers of 6–12 subunits that can be larger than 500 kD. The α- and β-CaM kinase isoforms are restricted to the nervous system whereas the γ- and δ-isoforms are present in essentially all tissues. Each gene may give rise to several isoforms by alternative splicing. Thus, α gives rise to α and α_{33} (α_B), β to β and β', γ to γ_A, γ_B and γ_C and δ to at least six isoforms (Bennett and Kennedy, 1987; Benson et al., 1991; Edman and Schulman, 1994; Mayer et al., 1993; Nghiem et al., 1993; Schworer et al., 1993).

Each CaM kinase isoform encodes a catalytic, regulatory, and association domain. The NH_2-terminal half of each isoform consists of the catalytic domain with inherent phosphotransferase activity. Under basal conditions, this activity is suppressed by a regulatory domain containing a kinase autoinhibitory segment and an overlapping calmodulin-binding segment which follow the catalytic domain. The COOH-terminal domain, termed the association/assembly domain, functions to assemble the subunits into a large multimeric structure and may localize the kinase within the cell by interaction with anchoring proteins. The predominant difference between the isoforms is their variable domain which consists of 11-39 amino acid "inserts" flanking a conserved linker sequence between the catalytic/regulatory and association domains. Electron microscopic studies suggest that in the multimeric holoenzyme, the catalytic/regulatory domains form individual spherical particles which radiate via a short tether from a single central globular particle formed by all the association domains (Kanaseki et al., 1991). Alternatively spliced sequences may be inserted on either the NH_2- or COOH-terminal side of the conserved linker segment forming the tether. Thus, inserts on the NH_2-terminal side may be physically closer to the catalytic/regulatory domain and could modify Ca^{2+}/calmodulin affinity or substrate specificity. Inserts on the COOH-terminal side would be part of the central globular particle and could serve to regulate the multimeric size of the kinase and its targeting to intracellular sites.

Targeting of CaM kinase to distinct subcellular sites may be achieved by a variety of strategies. These include posttranslational modification, targeting of mRNA followed by localized translation, and/or by alternative spliced inserts that direct differential localization within the cell. Some localization of α-CaM kinase may be achieved by local synthesis in dendrites. α-CaM kinase mRNA is found in dendrites as well as cell bodies of neurons whereas β-CaM kinase is restricted to cell bodies (Benson et al., 1992; Burgin et al., 1990). Posttranslational modification of α-CaM kinase may be responsible for some of its targeting since the same apparent isoform is found soluble and on synaptic vesicles (Benfenati et al., 1992).

Molecular cloning of additional CaM kinase isoforms provided us with an opportunity to determine whether isoforms differ in their intracellular localization and to examine the locus responsible for differential localization. We report that δ_B-CaM kinase, an isoform recently cloned from rat heart, is differentially targeted to the nucleus. By contrast, several other isoforms are excluded from the nucleus. An 11-amino acid insert present in the variable domain of δ_B-CaM kinase appears to be responsible for the nuclear targeting of CaM kinase.

Materials and Methods

Materials

Restriction enzymes and other DNA modifying enzymes were obtained from GIBCO BRL (Gaithersburg, MD) and New England Biolabs (Beverly, MA). Taq polymerase was obtained from Perkin-Elmer Cetus, (Norwalk, CT). PCR1000.12 cloning vector was obtained from In Vitrogen Corp (San Diego, CA). Antibodies and their sources were as follows: Anti-α-CaM kinase monoclonal antibody, CB-α-2 (mouse ascites) was generated in our laboratory as described previously (Baitinger et al., 1990); anti-haemagglutinin peptide (H) monoclonal antibody, 12CA5 was kindly provided by I. Wilson and is now available commercially through Berkeley Antibody Company (Richmond, CA); rhodamine-labeled anti-mouse affinity pure antibodies were obtained from Organo Technica Corp. (Durham, NC).

Methods

Construction of expression vectors. An oligonucleotide coding for the 18-amino acid haemagglutinin peptide (H) was introduced between Ser[3]

and Thr[4] at the amino-terminal end of δ_A-, δ_B-, and δ_C-CaM kinase, by oligonucleotide-directed mutagenesis (see below). The plasmid chosen for the expression of the wild-type, chimeric, and mutant CaM kinase constructs was the pCD derivative SRα (Takebe et al., 1988). δ-CAM kinases were introduced into the EcoRI sites of SRα.

Two unique restriction sites MluI and ScaI were inserted into α-CaM kinase (α-CaM kinase MS) by site-directed mutagenesis (see below) at amino acids 311 and 334, respectively. The cDNA encoding α-CaM kinase MS (1,512 bp including the EcoRI linkers) was introduced into the EcoRI site of PCR1000.12 vector to generate α-CaM kinase-PCR1000.12. δ_A-, δ_B-, and δ_C-CaM kinase sequences between amino acids 310 and 367, 310 and 344, and 310 and 333, respectively, were amplified by PCR (see below). The amplified sequences were introduced into α-CaM kinase-PCR 1000.12 replacing the 62-bp fragment between the MluI and ScaI sites thereby generating the chimeric α-(δ_A)-, α-(δ_B)-, and α-(δ_C)-CaM kinases. α-CaM kinase was inserted into SRα via the PstI–KpnI sites. The chimeric α-(δ)-CaM kinase-SRα and mutant chimeric α-(δ_B)-CaM kinase constructs were generated by replacing the central 1100 SmaI and BglII fragment of α-CaM kinase-SRα with the corresponding piece from α-(δ)-CaM kinase-PCR1000.12 and mutant α-(δ_B)-CaM kinase-M13 DNA, respectively.

Oligonucleotide-directed Mutagenesis

Site-directed mutagenesis of α- and α-(δ_B)-CaM kinase (as M13-CaM kinase) was performed as described previously for α-CaM kinase (Hanson and Schulman, 1992*a*; Waldmann et al., 1990). The α-(δ_B)-CaM kinase-M13 was constructed by subcloning the α-(δ_B)-CaM kinase cDNA into M13 at the EcoRI site. The following nucleotides were used to generate the MluI and ScaI sites without changing the respective amino acids in α-CaM kinase: MluI site at 928 bp 5′-CGGAGAAGTTACGCGTGGCCAGCATAG-3′ ScaI site at 990 bp 5′-GGTGGTGTTAGTACTCTCAGAGGATTC-3′. The following oligonucleotides were used to replace the indicated amino acids in α-(δ_B)-CaM kinase. Single mutants: Lys 328 to Asn 5′-CGAACTGGACTTCCTTTTGTTTACCCCATCCGG-3′; Lys 329 to Asn 5′-CGAACTGGACTTCCTGTTCTTTACCCCATCCGG-3′; Double mutant (Lys 328 to Asn; Lys 329 to Asn); 5′-CGAACTGGACTTCCTGTTGTTTACCCCATCCGG-3′ (See Fig. 1 *A* for amino acid numbering of mutants).

Site-directed mutagenesis was also used to "loop-in" a sequence coding for the 18-amino acid haemagglutinin peptide tag sequence (creating H-CaM kinase). The 90-mer oligonucleotide used had the sequence 5′-CCG GGT GCA GGT GGT GGT CAG CTG AGC TCC AGG TCC GGC GTA GTC AGG GAC GTC GTA CGG ATA AGG AGC TCC CGA AGC CAT GGC GGG GTC-3′ and introduced the amino acid sequence GAPYPYDVP-DYAGPGAQL between Ser[3] and Thr[4].

Mutations were identified by restriction analysis and confirmed by single strand DNA sequencing using the enzymatic dideoxynucleotide method with Sequenase (United States Biochem. Corp., Cleveland, OH). Double stranded mutant DNAs were subcloned into α-CaM kinase-SRα or α-(δ_Bl)-CaM kinase-SRα as described above.

PCR Amplification

All PCR reactions were carried out using the Twin Block thermal cycler system (Ericomp, Inc., San Diego, CA) and Taq polymerase. The reactions involved an initial denaturation period of 5 min at 94°C, followed by 25 cycles consisting of 1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C. Finally the amplification was completed with an additional cycle ending with a 10-min extension period. Sense oligo-nucleotides containing the MluI site and antisense oligonucleotides containing the ScaI site were used in pairs to amplify the isoform-specific variable sequence of δ_A-, δ_B-, and δ_C-CaM kinases.

Expression of CaM Kinase Constructs

COS-7 cells were maintained in DME with 10% supplemented calf serum (Hyclone Labs., Logan, UT) in a 10% CO_2 incubator. Rat 208F fibroblast cells were maintained in DME with 10% FBS (Hyclone) in a 10% CO_2 incubator. Calcium phosphate transfections were performed essentially as described previously (Chen and Okayama, 1987). DNA (10–15 μg per 10-cm plate containing ~70% confluent cells) was added as a coprecipitate with calcium phosphate in buffer containing 25 mM BES buffer, 140 mM NaCl, 130 mM $CaCl_2$, and 0.75 mM Na_2HPO_4 pH (6.95). Cells were mock transfected by the same procedure but received an SRα plasmid lacking the CaM kinase. Transfected cells were incubated in a 3% CO_2 incubator for 16–18 h, transferred to the 10% CO_2 incubator with a change of medium, and harvested 36–72 h later. For biochemical studies, the transfected cells were scraped into 10 ml of cold PBS, pelleted gently and resuspended in cold homogenization buffer (50 mM Pipes pH 7.0, 10% glycerol, 1 mM EGTA and a cocktail of the following protease inhibitors: Benzamidine 20 mM, PMSF 1 mM, leupeptin 10 μg/ml and pepstatin 10 μg/ml). Cells were disrupted by sonication in a water cup sonicator (Heat Systems-Ultrasonics) at maximal power for 40–60 s at 4°C and cell extracts were prepared by centrifugation in a microfuge at 13,000 *g* for 15 min. Aliquots were snap frozen in dry ice and stored at −70°C.

Culture and Transfection of Neonatal Cardiac Myocytes

Neonatal cardiac myocytes were prepared and cultured essentially as described previously (Sen et al., 1988) with a few modifications. Briefly whole hearts were isolated from 3-d old rats and minced in a balanced salt solution containing 0.2% glucose. The myocardial cells were dispersed using pancreatin and collagenase type II at 37°C, the supernatant was collected and the cells were isolated by centrifugation in a clinical centrifuge at 1,200 rpm for 5 min. The minced hearts were incubated with fresh collagenase and pancreatin and the myocardial cell isolation procedure repeated. The dispersion steps were repeated, for a total of four times. The cells were pooled and plated at a density of ~1.5×10^6 cells per 10-cm dish in MEM containing 10% horse serum, 5% FCS and 1% antibiotics. More than 95% of the cells stained positive for myosin indicating that very few fibroblasts were contaminating the myocyte culture. Cultured neonatal cardiac myocytes were transfected 48 h later by calcium phosphate precipitation as described above.

Immunocytofluorescence

Transfected 208F fibroblasts or neonatal cardiac myocytes were transferred 24–36 h later to polylysine-coated glass coverslips. After 12–15 h incubation on the coverslips, the cells were extensively rinsed in PBS and fixed in 2% HCHO in PBS for 10 min. The cells were further fixed and permeabilized with cold CH_3OH for 3–5 min. The fixed cells were extensively washed in PBS and preincubated in 1% BSA (RIA grade) in PBS for 1 h. The cells were further incubated with a primary antibody to either α-CaM kinase or the peptide tag (H), for 2 h at 37°C. The cells were extensively rinsed in PBS and incubated with goat anti-mouse antibody coupled to rhodamine for 45 min at 37°C. The coverslips were mounted on slides with vectashield mounting medium (Vector Labs., Burlingame, CA). The fluorescence was observed using a Zeiss Axioplan Universal microscope using 63× and 100× objectives.

Additional Methods

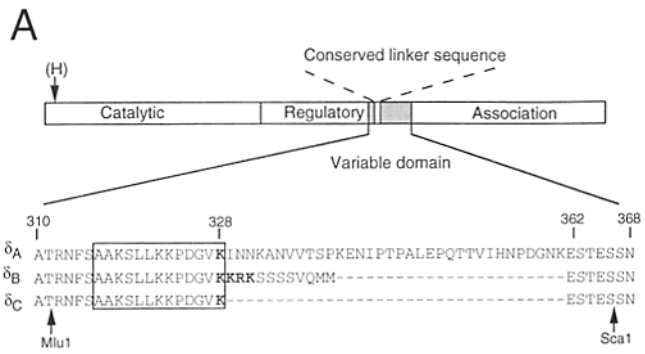
The activity of the transfected CaM kinases in the cell extract was assayed using a peptide substrate autocamtide 2 as described previously (Hanson et al., 1989). Assay conditions were similar to that previously described excepting that the specific activity of the [γ-^{32}P]ATP used was 1 Ci/mmole. Immunoblots were performed as previously described (Baitinger et al., 1990) using the anti-tag antibody or the anti-α-CaM kinase antibody and incubated for 2 h at room temperature at a dilution of 1:5,000 and 1:2,000, respectively.

Results

Generation and Expression of Epitope Tagged δ-CaM Kinase Isoforms

We chose to examine the effect of isoform-specific inserts on intracellular localization using the δ-CaM kinase isoforms. δ-CaM kinase is expressed as multiple alternatively spliced forms (Fig. 1, *A* and *B*) which do not differ in their basic physical and catalytic properties (Edman and Schulman, 1994). We examined δ_A-CaM kinase, which has a 34-amino acid insert on the COOH-terminal side of the linker segment of the variable domain, δ_B-CaM kinase, which has an 11-amino acid insert, and δ_C-CaM kinase, which lacks an insert in this position.

To detect the cellular distribution of the transfected δ-CaM



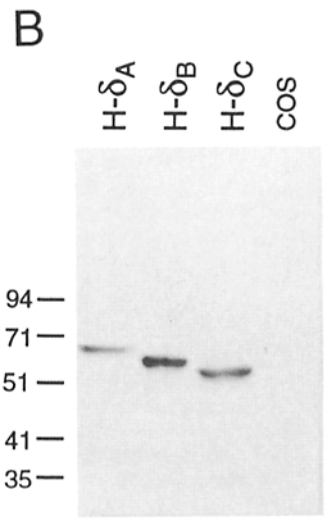


Figure 1. Variable domain of δ_A-, δ_B-, and δ_C-CaM kinases and the expression of tagged δ-CaM kinases (*H-δ_A-*, *H-δ_B-*, and *H-δ_C-CaM* kinases). (*A*) Amino acid sequence of the variable domain of δ_A-, δ_B-, and δ_C-CaM kinases and the generation of H-δ-CaM kinase constructs. The variable region amplified by PCR introduces the MluI and ScaI sites at the two ends of the amplified sequence at the positions indicated. The boxed amino acids represent the conserved linker sequence within the variable domain. The consensus NLS, KKRK, is highlighted in bold. The first lysine of the NLS (K^{328}) is the last residue of the linker sequence and is present in all the three δ-CaM kinases. The 18-amino acid HA peptide tag (*H*) was inserted at the NH_2-terminal of the three δ-CaM kinases, by mutagenesis as described in Materials and Methods. (*B*) Western blot analysis of the recombinant H-δ-CaM kinase proteins. Transfected COS cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose. The SRα transfected COS cell lysates were similarly immunoblotted as a control. The nitrocellulose blots were probed with anti-tag monoclonal antibody at a dilution of 1:5,000 as described in Materials and Methods. Immunoblotting of the H-δ-CaM kinase transfected COS-7 lysates indicates that the H-δ-CaM kinase subunits are specifically recognized by the anti-tag monoclonal antibody and migrate at expected positions on SDS polyacrylamide gel (*H-δ_A*-61 kD; *H-δ_B*-56 kD; *H-δ_C*-CaM kinase-54 kD).

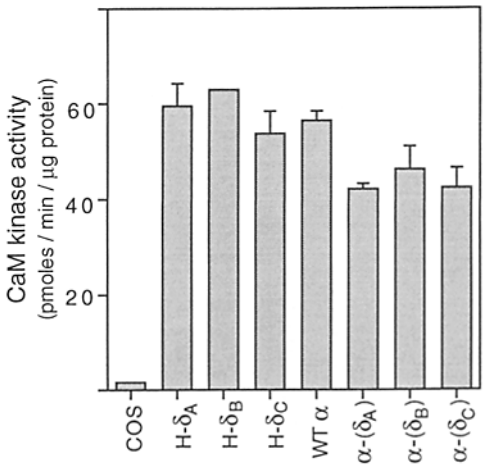


Figure 2. The H-δ-CaM kinase constructs and the chimeric α-(δ)-CaM kinase constructs exhibit normal enzyme activity. COS cell lysates from cells transfected with α-CaM kinase, δ-CaM kinases containing the 18-amino acid haemagglutinin peptide-tag at their amino terminal end (H-δ_A, H-δ_B-, H-δ_C), or the chimeric α-(δ)-CaM kinases (α-(δ_A)-, α-(δ_B)-, α-(δ_C)-) were assayed for CaM kinase activity as described in Materials and Methods. The control or endogenous CaM kinase activity in COS cells was assayed in lysates from COS cells transfected with the SRα expression vector alone.

kinases, we introduced an epitope tag into each one. An 18-amino acid segment containing a sequence from hemagglutinin HA1 that constitutes the antigenic epitope for monoclonal antibody 12CA5, was introduced by site-directed mutagenesis into the three δ-CaM kinases between Ser^3 and Thr^4, to generate the tagged constructs H-δ_A-CaM kinase, H-δ_B-CaM kinase, and H-δ_C-CaM kinase (Field et al., 1988; Wilson et al., 1984). We checked the basic properties of the three epitope tagged δ-CaM kinases in the SRα vector expressed in COS cells (Materials and Methods). Western blotting of SDS-PAGE of COS cell extracts using anti-tag antibody indicates that the three tagged kinases were expressed with a subunit size ~2 kD larger than the corresponding subunit lacking the tag (H-δ_A, 61 kD; H-δ_B, 56 kD; H-δ_C, 54 kD) (Fig. 1 *B*). Little or no proteolytic products could be seen in these transfections. Furthermore, only a single prominent band was seen in each lane of the transfected COS cell extracts and no immunoreactivity was seen in the control extract, attesting to the lack of significant cross-reacting antigen in these cells. The presence of the epitope tag did not affect kinase function. Each isoform was expressed as well as α-CaM kinase, a well characterized neuronal isoform (Fig. 2). Ca^{2+}/calmodulin-dependent phosphorylation of autocamtide-2, a selective CaM kinase substrate, was increased 20–30-fold relative to mock-transfected cells. Kinase activity was highly dependent on both Ca^{2+} and calmodulin. CaM kinase activity migrated on sucrose gradients as a characteristic multimeric holoenzyme of ~500 kD (data not shown) as previously found with the non-tagged isoforms (Edman and Schulman, 1994).

Immunolocalization of H-δ_A, H-δ_B, and H-δ_C-CaM Kinase Transfected into 208F Fibroblasts

The three tagged constructs, in the SRα expression vector, were transfected into 208F cells (fibroblasts) in order to determine whether alternative splicing affects the intracellular targeting of δ-CaM kinase. Cells were fixed 36 h after transfection and incubated with the anti-tag antibody (Fig. 3). A goat anti-mouse IgG coupled to rhodamine was used to detect the mouse monoclonal antibody by fluorescent microscopy. Approximately 20–40% of the cells exhibited immunofluorescence, indicating a transfection efficiency of 20–40%. Expression of H-δ_A-CaM kinase and H-δ_C-CaM kinase resulted in a cytosolic immunofluorescence pattern, suggesting that these isoforms were cytosolic or cytoskeletal (Fig. 3, *a* and *b*). There was little or no immunofluorescence in the nucleus. By contrast, all the immunofluorescence in H-δ_B-CaM kinase expressing cells was nuclear (Fig. 3, *c*

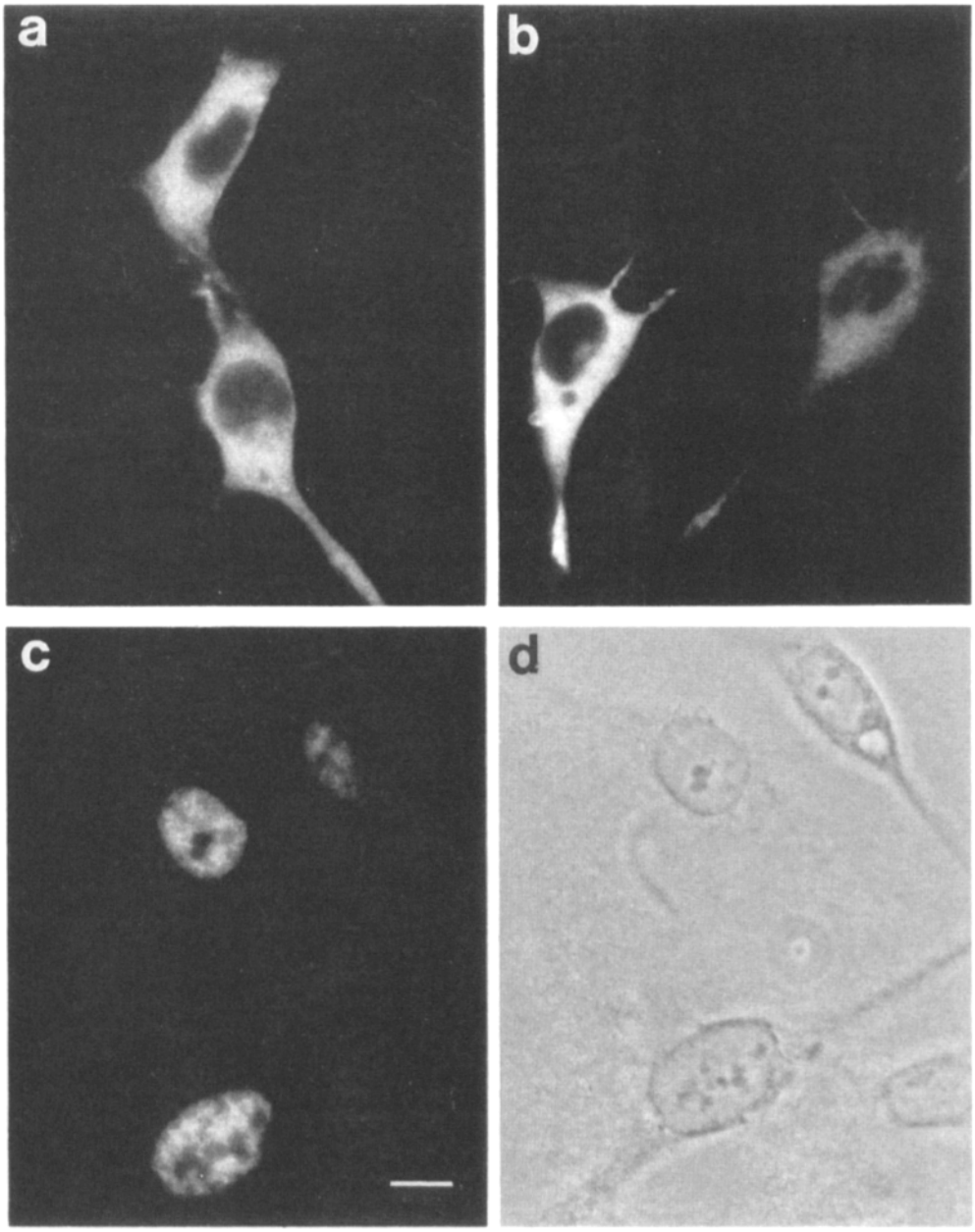


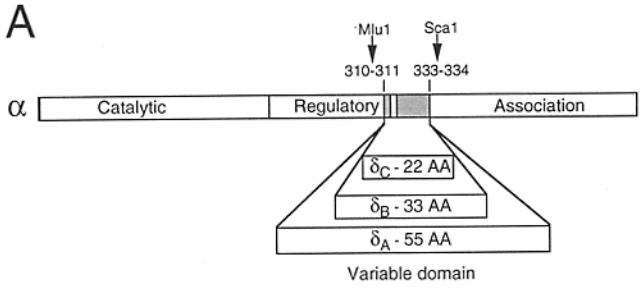
Figure 3. δ_B-CaM kinase is targeted to the nucleus whereas δ_A- and δ_C- are localized to the cytosol in transfected 208F cells. 208F cells (*rat fibroblasts*) transfected with H-δ_A-, H-δ_B-, H-δ_C-CaM kinase constructs were fixed and analyzed by indirect immunofluorescence using the anti-tag antibody as described in Materials and Methods. The efficiency of transfection was ~20–40%. (*a*) H-δ_A-CaM kinase–transfected cells; (*b*) H-δ_C-CaM kinase–transfected cells; (*c*) H-δ_B-CaM kinase–transfected cells; (*d*) phase contrast of specimen (*c*) in the same field showing the entire set of cells with their nuclei. Bar, 20 μm.

and *d*). Thus, the transfected H-δ_B-CaM kinase was almost exclusively targeted to the nucleus. The transfected cells exhibited varying degrees of CaM kinase expression, but in all cases the pattern of staining was identical in that the H-δ_B-CaM kinase was nuclear whereas H-δ_A- and H-δ_C-CaM kinase were cytoplasmic.

Generation and Expression of Chimeric CaM Kinases

Does the δ_B-CaM kinase-specific insert encode nuclear targeting information? The only differences in sequence between the three δ-CaM kinase isoforms are the isoform-specific inserts present in δ_A- and δ_B-CaM kinases and absent in δ_C-CaM kinase (Fig. 1 *A*). We therefore engineered chimeric kinases in which the δ_A- and δ_B-specific sequences were introduced in the corresponding position of α-CaM kinase. Like δ_C-CaM kinase, α-CaM kinase lacks inserts at both the NH_2- and COOH-terminal sides of the conserved linker sequence and is normally not targeted to the nucleus (see below). Furthermore, α-CaM kinase is the best characterized CaM kinase and an isoform for which a specific antibody is available.

We prepared CaM kinase chimeras to determine whether the δ-specific sequences would reroute a normally cytosolic/cytoskeletal α-CaM kinase. Unique restriction sites were introduced on either site of the variable region of α-CaM kinase by site-directed mutagenesis (Fig. 4 *A*). The variable regions of δ_A-, δ_B, and δ_C-CaM kinases were amplified by PCR using primers that included the identical restriction sites at comparable positions (Fig. 1 *A*). This enabled us to generate chimeras with the variable domains of the three δ-CaM kinase isoforms inserted into an identical position of α-CaM kinase (Fig. 4 *A*). Expression of the resulting chimeric constructs (referred to from here on as



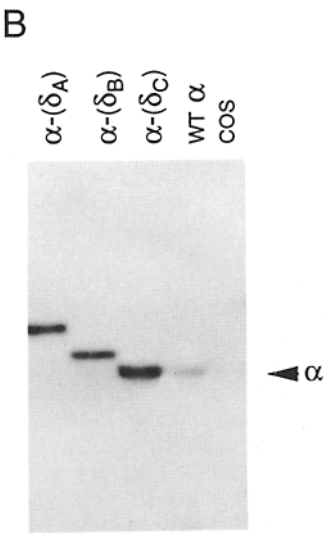


Figure 4. Generation and expression of chimeric α-(δ)-CaM kinases. (*A*) Generation of chimeric α-(δ)-CaM kinase constructs. MIu1 and Sca1 sites were inserted into α-CaM kinase at positions identical to that of δ-CaM kinase, by site-directed mutagenesis. The PCR amplified variable sequences of δ_A-, δ_B-, and δ_C-CaM kinases (Fig. 1 *A*) were inserted individually in place of the α-CaM kinase variable sequence via the MIu1 and Sca1 sites (Materials and Methods). (*B*) Western blot analysis of chimeric α-(δ)-CaM kinase proteins. Transfected COS cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose. The SRα transfected COS cell lysates were similarly immunoblotted as a control. The nitrocellulose blots were probed with anti-α-CaM kinase monoclonal antibody at a dilution of 1:2,000 as described in Materials and Methods. Immunoblotting of the chimeric α-(δ)-CaM kinase transfected COS-7 lysates with the anti-α-CaM kinase monoclonal antibody demonstrates that the chimeric CaM kinases are recognized by the anti-α-CaM kinase antibody. They migrate on SDS-polyacrylamide gels, at positions predicted by the replacement of the α variable region by the δ_A-, δ_B, δ_C-variable regions.

α-(δ_A)-CaM kinase, α-(δ_B)-CaM kinase, and α-(δ_C)-CaM kinase) was first examined by transfecting the constructs into COS cells. The protein products were of the expected size and were specifically recognized by a monoclonal antibody to α-CaM kinase (Fig. 4 *B*). Each chimera produced a protein kinase which was fully dependent on Ca^{2+}/calmodulin for activity (Fig. 2). The level of expression was comparable to wild-type α-CaM kinase.

Intracellular Targeting of Chimeric α-(δ)-CaM Kinases

α-CaM kinase and the three chimeras were expressed in 208F cells and their intracellular targeting was examined with the anti-α-CaM kinase antibody. Native α-CaM kinase that lacked any δ-CaM kinase sequences was excluded from the nucleus and was broadly distributed within the cell to the cytosol/cytoskeleton (Fig. 5 *a*). Similarly, α-(δ_A)- and α-(δ_C)-CaM kinases were cytoplasmic, with little immunofluorescence seen in the nucleus (Fig. 5, *b* and *e*). The α-(δ_B)-CaM kinase exhibited a distinctly nuclear localization with little or no immunofluorescence detected in the cytoplasm despite the high level of expression (Fig. 5, *c* and *d*). Comparison of the amino acid sequences of α-(δ_C)-CaM kinase (which has 23 amino acids derived from δ_C-CaM kinase) and α-(δ_B)-CaM kinase (which has 34 amino acids derived from δ_B-CaM kinase) shows that there is an 11-amino acid insert unique to δ_B-CaM kinase (Fig. 1 *A*). Since this insert is sufficient to reroute a cytoplasmic α-CaM kinase to the nucleus, it is likely that it encodes information for nuclear targeting which functions independent of the rest of the δ_B-CaM kinase sequence.

Nuclear targeting of δ_B-CaM kinase and α-(δ_B)-CaM kinase does not appear to result from the generation of truncated fragments or unassembled subunits that would be small enough to diffuse passively into the nucleus. We needed to exclude such a possibility since truncated CaM kinase constructs, which lack the association domain, like α-CaM kinase (1-326), are expressed as monomers of ~30–35 kD. These monomers express Ca^{2+}/calmodulin-dependent kinase activity and are small enough to passively diffuse into the nucleus (unpublished observations). Several properties of α-(δ_B)-CaM kinase suggest that its nuclear targeting is not due to such an artifact. First, the subunit size of the expressed α-(δ_B)-CaM kinase is commensurate with the size expected of full-length α(δ_B)-CaM kinase. A Western blot of cell extracts resolved on SDS-PAGE using the same α-CaM kinase-specific antibody used for immunofluorescence does not detect smaller fragments (Fig. 4 *B*). Second, Ca^{2+}/calmodulin-stimulated phosphorylation of extracts from transfected cells reveals a predominant autophosphorylated 56-kD protein corresponding to a full-length α-(δ_B)-CaM kinase (data not shown). Third, expressed α-(δ_B)-CaM kinase assembles appropriately into multimeric holoenzymes as determined by sucrose density centrifugation (data not shown). At least 95% of CaM kinase activity sediments as a peak of ~500 kD with little activity in fractions sedimenting at the monomer size. Even if a significant fraction of α-(δ_B)-CaM kinase failed to assemble or was made smaller by truncation, it would still not give such a strikingly selective localization in the nucleus as seen above (Fig. 5 *c*).

The δ_B-Specific Insert Has a Nuclear Localization Signal

Comparison of the 11-amino acid insert in δ_B-CaM kinase with established nuclear localization signal (NLS) sequences of known nuclear proteins reveals that it has an NLS similar to that of SV40 T antigen (SV40 T Ag). The core NLS of the SV40 T Ag, ^{128}KKRK (Kalderon et al., 1984*a*), is found identically in the δ_B-CaM kinase-specific insert as ^{328}KKRK (Fig. 6). Does this sequence serve as the NLS that is responsible for nuclear localization of δ_B-CaM kinase? Extensive studies of NLS sequences indicate that the first Lys residue in the sequence KKRK is critical for nuclear targeting. For example, mutation of this Lys in the NLS of SV40 T Ag to Asn or Thr disabled nuclear targeting via the NLS and led to the expression of the construct in the cytosol (Lanford and Butel, 1984; Kalderon et al., 1984*a*). We therefore examined the role of the Lys residues in ^{328}KKRK of δ_B-CaM kinase by generating site-directed mutants of α-(δ_B)-CaM kinase in which either the first, second, or first two Lys residues in the KKRK sequence were substituted

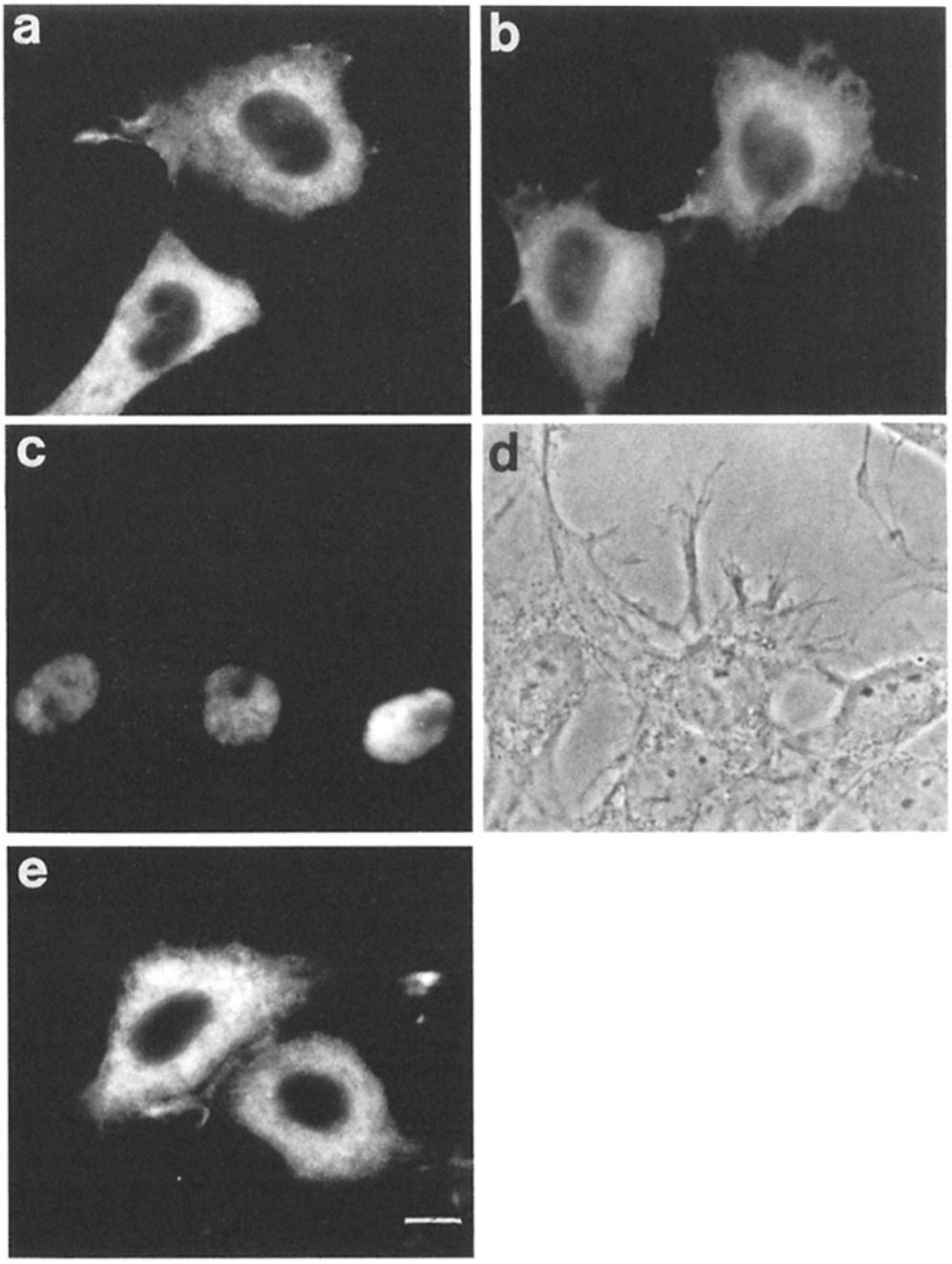


Figure 5. The variable domain of δ_B- encodes a nuclear local signal (*NLS*) which targets a cytosolic α-CaM kinase to the nucleus. 208F cells transfected with wild-type α-CaM kinase or chimeric α-(δ_A)-, α-(δ_B)-, or α-(δ_C)-CaM kinase were fixed and analyzed by indirect immunofluorescence using the anti-α CaM kinase antibody (*CB-α-2*) as described in Materials and Methods. The transfection efficiency was ~20–40%. (*a*) α-CaM kinase–transfected cells; (*b*) α-(δ_A)-CaM kinase–transfected cells; (*c*) α-(δ_B)-CaM kinase–transfected cells; (*d*) phase contrast of *c* in the same field; (*e*) α-(δ_C)-CaM kinase–transfected cells. Bar, 20 μm.

SV40 T Ag	-TPPK**KKRK**VEDPLA-
δ_B-CaM kinase	-PDGV**KKRK**SSSSVQMM----------EST
α_B-CaM kinase	-NDGV**KKRK**SSSSVQLM----------EST
γ_A-CaM kinase	-DGGV**KKRK**SSSSVHLMEQTTVVHNATGIKGSTEST
δ_B (Lys^{328Asn})	-PDGV**NKRK**SSSSVQMMEST-
δ_B (Lys^{329Asn})	-PDGV**KNRK**SSSSVQMMEST-
δ_B ($Lys^{328Asn}Lys^{329Asn}$)	-PDGV**NNRK**SSSSVQMMEST-
	328

Figure 6. Comparison of the insert sequence in the variable domains of δ_B-, α_B-, and γ_A-CaM kinases with that of the SV40 T Ag NLS. The consensus NLS, KKRK, is highlighted in bold. The CaM kinase isoform-specific sequences in the variable domain, which are likely inserted by alternative splicing, are enclosed in a dashed box. The insertion of the isoform specific sequences generates the NLS sequence, KKRK, in δ_B-, α_B-, and γ_A-CaM kinase isoforms. The two lysines of the δ_B-CaM kinase NLS which were mutated to Asn are Lys^{328} and Lys^{329}. The mutant δ_B-CaM kinase NLS sequences (δ_B- Lys^{328Asn}, δ_B-Lys^{329Asn}, δ_B-$Lys^{328Asn}Lys^{329Asn}$) are highlighted in bold.

with Asn. We shall use the convention of referring to these mutations as Lys^{328Asn}, Lys^{329Asn}, or $Lys^{328Asn}Lys^{329Asn}$, respectively (Fig. 6). As before, each of these mutated α-(δ_B)-CaM kinase chimeras were first characterized in COS cells. All three mutant proteins were expressed at comparable levels and demonstrated the characteristic Ca^{2+}/calmodulin-

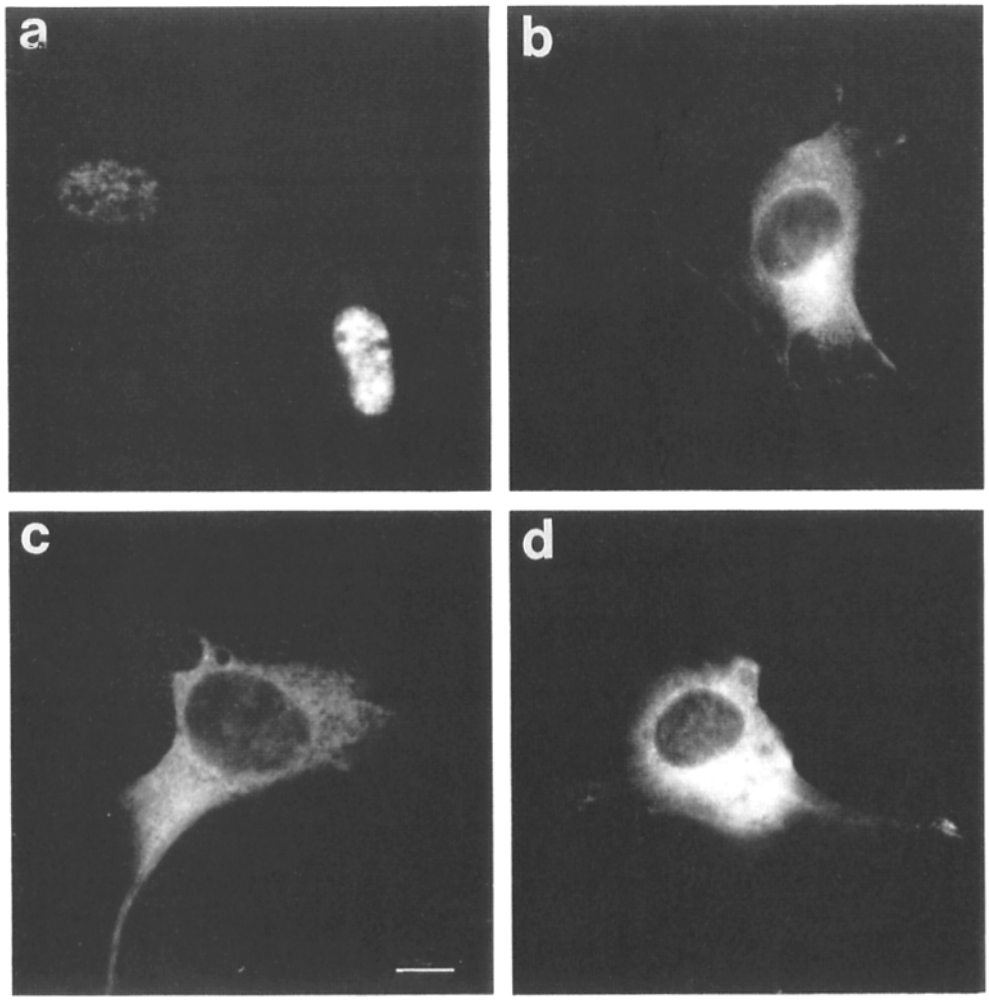


Figure 7. Mutagenesis of the first or the second lysine of the putative NLS prevents the nuclear targeting of CaM kinase. Fibroblasts transfected with α-(δ_B)-CaM kinase or mutant α-(δ_B)-CaM kinases were fixed and analyzed by indirect immunofluorescence using the CB-α-2 antibody. (*a*) α-(δ_B)-CaM kinase–transfected cells; (*b*) α-(δ_B)-CaM kinase-[Lys^{328Asn}]–transfected cells; (*c*) α-(δ_B)-CaM kinase-[Lys^{329Asn}]–transfected cells; (*d*) α-(δ_B)-CaM kinase-[$Lys^{328Asn}Lys^{329Asn}$]–transfected cells. Bar, 20 μm.

dependent CaM kinase activity (data not shown). Both the sequencing and functional expression of the mutants indicated that the mutant CaM kinase proteins were full-length.

The α-(δ_B)-CaM kinase chimera, with and without mutations in the putative NLS, were transfected into 208F cells and their intracellular targeting was examined by immunolocalization as above. As previously observed, α-(δ_B)-CaM kinase was exclusively nuclear (Fig. 7 *a*). By contrast, mutation of the Lys^{328} to Asn completely blocked the entry of α-(δ_B)-CaM kinase into the nucleus (Fig. 7 *b*). The Lys^{328Asn} mutant could not be detected in the nucleus even after 72 h after transfection, thus clearly indicating that the mutation prevents, rather than just slows down the nuclear import. Interestingly, the Lys^{329Asn} mutant was also excluded from the nucleus as was the double mutant Lys^{328Asn} Lyn^{329Asn}. These experiments strongly suggest that this site in δ_B-CAM kinase functions as a NLS sequence that is critical for targeting the kinase to the nucleus. Unlike the NLS of SV40 T Ag, both the first and second lysine residues are necessary for nuclear targeting of CaM kinase.

δ_B-CaM Kinase Is Targeted to the Nucleus in Cardiac Myocytes

We determined the intracellular targeting of H-δ_B-, and H-δ_C-CaM kinase in cardiac myocytes, since the cDNAs for these isoforms were isolated from a heart library (Edman and Schulman, 1994). Although δ_B-CaM kinase is targeted to the nucleus in fibroblasts, it is possible that in cardiac cells its NLS is masked or overridden by the anchoring of the kinase to a cytoskeletal or cytosolic protein. We prepared cardiac myocytes from neonatal rats, transfected them with the H-δ_A-, H-δ_B-, and H-δ_C-CaM kinase constructs, and followed their localization with the anti-tag antibody as above. δ_B-CaM kinase was clearly targeted to the myocyte nucleus (Fig. 8 *c*) whereas the δ_A- and δ_C-CaM kinase were excluded from the nucleus and localized to the cytosol/cytoskeleton (Fig. 8, *a* and *b*). We also examined the cellular targeting of the chimeric constructs in the cardiac myocytes. As expected, the H-α-(δ_B)-CaM kinase chimera was targeted exclusively to the nucleus whereas H-α-(δ_A)- and H-α-(δ_C)-CaM kinase were localized to the cytoplasm (data not shown).

Targeting of CaM Kinase Is Determined by the Ratio of Nuclear to Non-Nuclear Isoforms Expressed in the Cell

CaM kinase may consist of holoenzymes with subunits which encode distinct targeting information. This is likely to occur since a number of cell types have been shown to express more than one isoform. Neurons express at least the

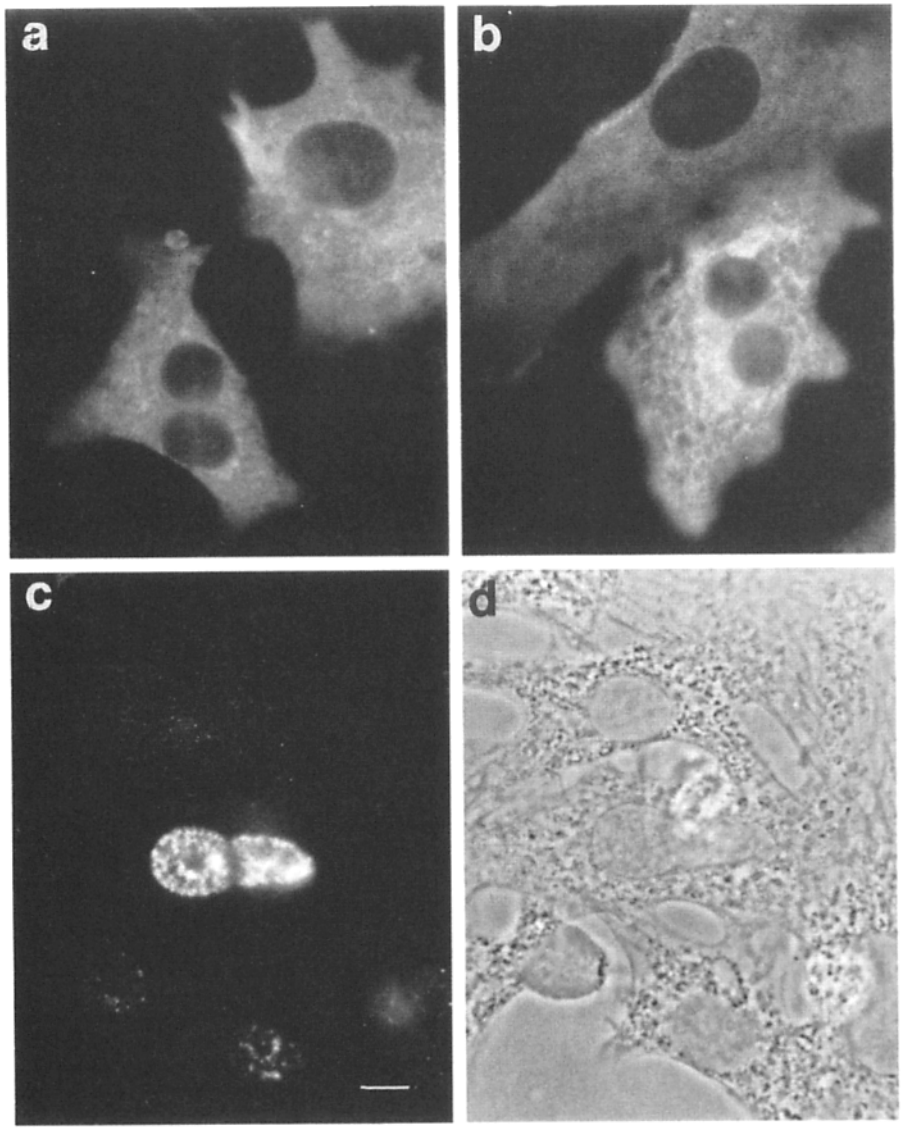


Figure 8. δ_B-CaM kinase is targeted to the nucleus in transfected primary rat cardiac myocytes. Neonatal rat cardiac myocytes prepared as described in Materials and Methods were transfected with H-δ_A-, H-δ_B-, or H-δ_C-CaM kinase construct. 48 h after transfection, the cells were fixed and analyzed by indirect immunofluorescence using the anti-tag antibody. (*a*) H-δ_A-CaM kinase-transfected cells; (*b*) H-δ_C-CaM kinase-transfected cells; (*c*) H-δ_B-CaM kinase-transfected cells; (*d*) phase contrast of *c* in the same field. Bar, 20 μm.

α and β isoforms of CaM kinase (Scholz et al., 1988), lymphocytes have at least two γ isoforms and one δ isoform (Nghiem et al., 1993) and epithelial cells seem to express both γ and δ isoforms (Nghiem et al., 1993). Coexpression of α- and β-CaM kinase cDNAs in CHO cells leads to the coassembly of α- and β-CaM kinase subunits to form a holoenzyme (Yamauchi et al., 1989). Nuclear and non-nuclear isoforms may therefore assemble as heteromultimers and modulate intracellular localization of the two isoforms.

We therefore asked whether nuclear and non-nuclear isoforms can coassemble, whether coassembly occurs before targeting, and whether coassembly affects targeting. We cotransfected various pairwise combinations of nuclear and non-nuclear isoforms in which only one member of any pair contained the epitope tag or could be distinguished by an isoform-specific antibody. Four pairs were tested—H-α-(δ_B)-CaM kinase with α-CaM kinase, α-(δ_B)-CaM kinase with H-α-CaM kinase, δ_B-CaM kinase with α-CaM kinase (distinguishable by α-CaM kinase specific antibody), and H-δ_C-CaM kinase with δ_B-CaM kinase.

We cotransfected H-δ_C-CaM kinase (cytosolic isoform) with a large excess (20-fold) of δ_B-CaM kinase (nuclear isoform) into 208F cells and determined the cellular localization of H-δ_C-CaM kinase using the anti-tag antibody. Interestingly, greater than 99% of the transfected cells expressed H-δ_C-CaM kinase in the nucleus (Fig. 9, *a* and *b*). Since δ_C-CaM kinase does not encode a NLS and is localized to the cytoplasm when expressed in the absence of any other isoform (Fig. 3 *b*), H-δ_C-CaM kinase is likely coassembling with the nuclear δ_B-CaM kinase and the holoenzyme is then being transported into the nucleus by the NLS in the δ_B-subunits. We obtained an identical result when we cotransfected α-CaM kinase with an excess of δ_B-CaM kinase. The cytosolic α-CaM kinase was rerouted by the nuclear δ_B-CaM kinase subunits and localized almost exclusively in the nucleus (Fig. 9, *c* and *d*). We observed a similar pattern of cellular staining when an excess of the chimeric α-(δ_B)-CaM kinase (nuclear isoform) was cotransfected with the H-α-CaM kinase (cytosolic isoform). The H-α-CaM kinase was expressed in the nucleus of transfected

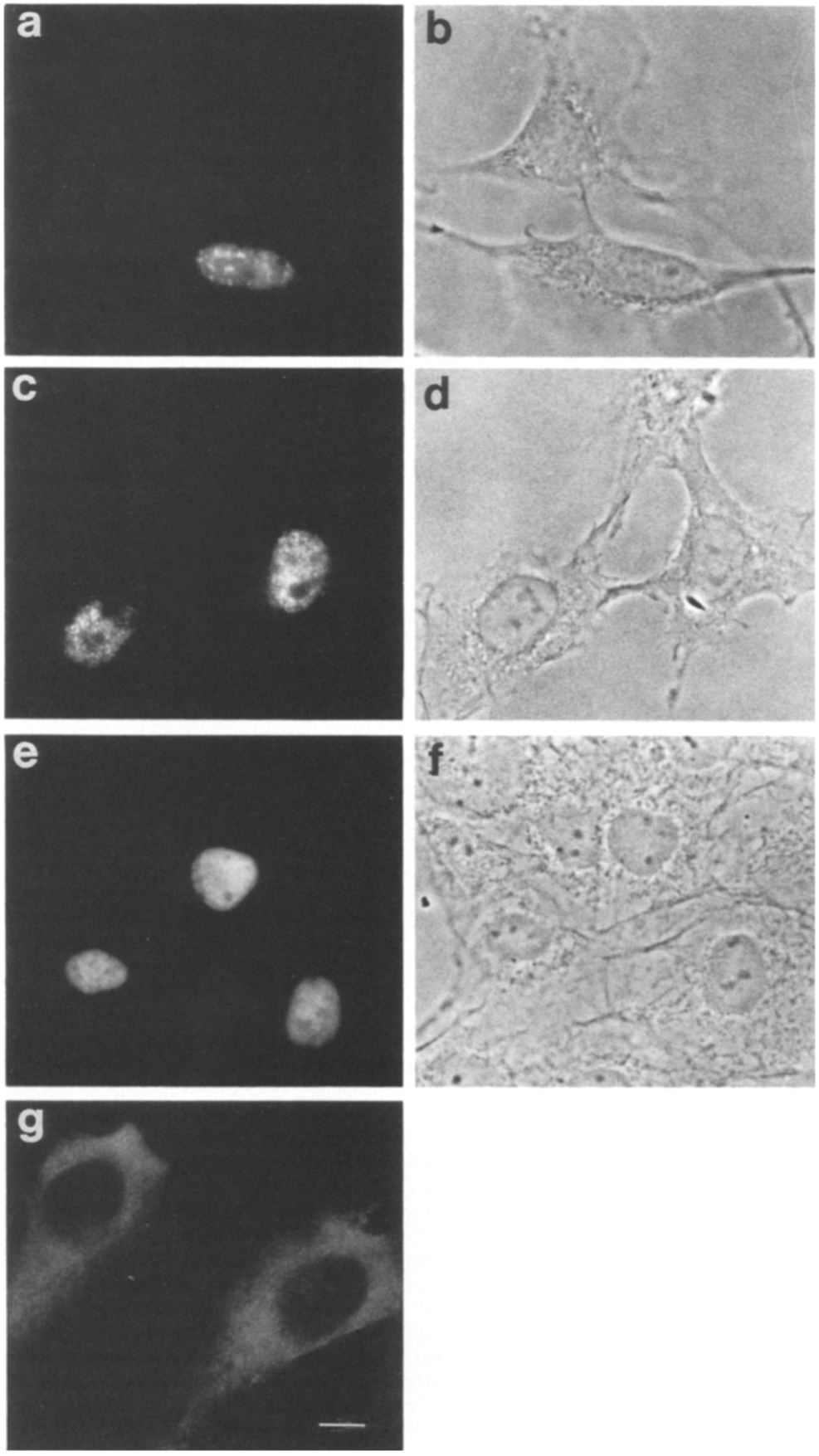


Figure 9. Ratio of the nuclear to the non-nuclear isoform of CaM kinase expressed in a cell, determines the intracellular targeting of both the isoforms of CaM kinase. 208F cells cotransfected with a nuclear and a non-nuclear isoform of CaM kinase cDNA at 1:20 or 20:1 ratio by weight, were fixed and analyzed by indirect immunofluorescence using the anti-tag antibody (*a*, *b*, *e*, *f*, and *g*) or anti-α-CaM kinase monoclonal antibody (*c* and *d*) (see Materials and Methods). Cells were transfected with the following CaM kinase constructs: (*a* and *b*) δ_B:H-δ_C at 20:1; (*c* and *d*) δ_B:α at 20:1; (*e* and *f*) α (δ_B):H-α at 20:1; (*g*) H-α-(δ_B):α at 1:20. (*b*, *d*, and *f*) phase contrast of *a*, *c*, and *e*, respectively. Bar, 20 μm.

cells as determined by anti-H antibody staining (Fig. 9, *e* and *f*). Thus, an NLS-bearing isoform, either as a chimera or as a naturally occurring isoform can target the entire holoenzyme to the nucleus when it is in excess of the coassembled non-nuclear isoforms.

Cytosolic isoforms determine the cellular targeting when they are the dominant isoform subunits in the holoenzyme. Transfection of a large excess (20-fold) of α-CaM kinase (cytosolic isoform) with H-α-(δ_B)-CaM kinase (nuclear isoform) results in the localization of H-α-(δ_B)-CaM kinase in the cytosol in more than 99% of the transfected cells (Fig. 9 *g*). A similar pattern of cellular distribution was observed when either δ_C-CaM kinase or α-CaM kinase was transfected at a 20:1 ratio to H-δ_B-CaM kinase (data not shown). These experiments indicate that CaM kinase isoforms can coassemble as heteromultimers and that the numerically dominant subunit in the holoenzyme determines its cellular targeting.

Coexpression of the nuclear and the non-nuclear isoforms in intermediate ratios leads to an intermediate distribution of the holoenzyme within the cell. At a 10:1 ratio of α-(δ_B)-CaM kinase to H-α-CaM kinase, 91% of the transfected cells expressed the cytosolic isoform in the nucleus (Table I). Decreasing the ratio of α-(δ_B)-CaM kinase to α-CaM kinase led to the localization of α-CaM kinase in both the cytoplasm and the nucleus. Thus, at a ratio of 1:1, less than 2% of the transfected cells expressed H-α-CaM kinase exclusively in the nucleus while 67% of the cells expressed H-α-CaM kinase equally in the nucleus and in the cytoplasm. Conversely, as the ratio of α-CaM kinase to H-α-(δ_B)-CaM kinase was increased, none of the transfected cells expressed CaM kinase in the nucleus and an increasing number of cells expressed the nuclear H-α-(δ_B)-CaM kinase isoform in the cytosol (Table I). For example at a 5:1 ratio of α-CaM kinase to H-α-(δ_B)-CaM kinase, none of the transfected cells expressed the H-α-(δ_B)-CaM kinase in the nucleus while 16.5% of the cells expressed the nuclear H-α-(δ_B)-CaM kinase exclusively in the cytoplasm. These experiments clearly demonstrate that the ratio of the two isoforms in a holoenzyme can determine the intracellular distribution of the CaM kinase holoenzyme.

Table I. Cellular Distribution of CaM Kinase Protein after Coexpression of Nuclear and Non-nuclear Isoforms of CaM Kinase (Percentage of CaM Kinase Expressing Cells)

α-(δ_B):α	N	N > C	N ≈ C	N < C	C
10:1	91.3	8.7	0	0	0
5:1	68.6	27.6	3.8	0	0
2.5:1	26.5	50.3	23.2	0	0
1:1	1.8	31.1	67.1	0	0
1:2.5	0	4.8	28.5	63.5	3.2
1:5	0	0	4.7	78.8	16.5
1:10	0	0	6.1	10.2	83.7

N, (% cells exhibiting nuclear staining); *C*, (% cells exhibiting cytoplasmic staining). Varying ratios of α-(δ_B)-CaM kinase (nuclear) and α-CaM kinase (cytoplasmic) were cotransfected into 208F cells. In the first four rows of the table, α-CaM kinase was tagged with a haemagglutinin peptide (H) in order to specifically follow the cellular distribution of the α-CaM kinase protein. In the last three rows the α-(δ_B)-CaM kinase was tagged with the haemagglutinin peptide to follow the cellular localization of α-(δ_B)-CaM kinase. The number of transfected cells scanned per experiment was between 100 and 150. The number of cells showing exclusively nuclear (*N*), more nuclear than cytoplasmic (N > C), approximately equally nuclear and cytoplasmic (N ≈ C), more cytoplasmic than nucler (N < C) or exclusively cytoplasmic (*C*) staining with the anti-tag (*H*) monoclonal antibody, were expressed as a percentage of the total number of transfected cells examined. The scoring of the transfected cells was performed in a blind fashion.

Discussion

This paper directly demonstrates isoform-specific targeting of multifunctional CaM kinase. The enzyme has been known to localize at a variety of intracellular sites and it seemed reasonable to expect that its localization in a given cell would depend on the specific isoform being expressed and the complement of anchoring proteins that the kinase may bind (Rostas and Dunkley, 1992). Its differential concentration in postsynaptic density from forebrain and cerebellum was postulated to be a result of targeting of the kinase to this neuronal specialization by the α-CaM kinase isoform (Miller and Kennedy, 1985). We approached this issue directly by testing the targeting of distinct CaM kinase isoforms introduced into cells by transfection and identified a nuclear isoform of the enzyme. We show that an NLS in a 11-amino acid insert sequence at the beginning of the association domain of the δ-CaM kinase is responsible for targeting this isoform, δ_B-CaM kinase, to the nucleus. This NLS is similar to the NLS of the SV40 T Ag and fits the NLS consensus sequence, K-K/R-X-K/R, proposed by Chelsky and coworkers (Chelsky et al., 1989; Garcia-Bustos et al., 1991). We have demonstrated that the first two lysines of the NLS are critical for nuclear targeting of CaM kinase as their replacement by asparagine, a neutral amino acid, prevents the nuclear entry of CaM kinase. This NLS acts independently of any distal sequences of the δ-CaM kinase isoform since a chimeric construct of α-CaM kinase which contains the NLS from the δ_B-CaM kinase is rerouted from the cytosol to the nucleus. In cardiac myocytes, which express endogenous δ_B-CaM kinase, the transfected δ_B-CaM kinase is targeted to the nucleus, suggesting that in its native environment the NLS targets δ_B-CaM kinase to the nucleus. Finally, we observe that the intracellular targeting of the nuclear isoform is modified by coassembly with other isoforms of CaM kinase, generating a heteromultimeric holoenzyme.

The first lysine of the NLS is present in the δ_C-CaM kinase isoform which lacks any insert sequence and has the sequence KEST (Fig. 1 *A*). In the δ_B-CaM kinase isoform, the 11-amino acid insert sequence which begins with KRK is inserted between the residues K and E of the sequence KEST. The insert sequences that constitute the variable domain of CaM kinase are likely introduced by alternative splicing. In the case of δ_B-CaM kinase the insert sequence, **KRKSSSSVQMM**, is introduced after the last lysine of the previous exon, thus generating the sequence **KKRKSSSSV**-QMM (Fig. 1 *A*). It therefore appears that alternative splicing generates the NLS sequence, **KKRK**, which is responsible for the nuclear targeting of δ_B-CaM kinase.

Previous studies, based on kinase activity, immunoreactivity or function suggested the presence of CaM kinase in the nucleus. Sahyoun and coworkers (Sahyoun et al., 1984*a*, *b*) found a CaM kinase activity in the nucleus which phosphorylated nuclear matrix proteins and chromatin proteins like HMG17, proteins that have been implicated in the regulation of RNA transcription. Ohta et al. (1990) presented immunocytochemical evidence for a nuclear localization of

CaM kinase in fibroblasts and glioma cells (Ohta et al., 1990). CaM kinase has also been implicated in regulating various nuclear functions including nuclear envelope breakdown, G2-M transition in mitosis and gene expression (Baitinger et al., 1990; Planas-Silva and Means, 1992; Sheng et al., 1991; Wegner et al., 1992). The isoform-specific targeting of CaM kinase to the nucleus reported here specifically identifies which isoforms may be present in the nucleus and suggests a possible mechanism by which targeting is achieved.

The NLS of the SV40 T Ag, of which the δ_B-CaM kinase NLS is a prototype, has been extensively analyzed. Peptides and mutant constructs with this minimal NLS sequence have been demonstrated to target a variety of reporter proteins to the nucleus (Chelsky et al., 1989; Lanford et al., 1986; Kalderon et al., 1984*b*). Recently NLS-binding proteins have been identified which bind and transport the NLS-containing proteins into the nucleus (Adam et al., 1989; Adam and Gerace, 1991). The NLS has been shown to function independent of its position in the protein (Kalderon et al., 1984*b*). The first lysine residue of the NLS (KKRK) is critical for the nuclear targeting of the SV40 T Ag (Kalderon et al., 1984*a*; Lanford and Butel, 1984). Nuclear proteins more commonly use a bipartite NLS exemplified by nucleoplasmin in which two clusters of positively charged residues separated by ten random amino acids are involved (Dingwall and Laskey, 1991; Robbins et al., 1991). Mutation of residues in any one cluster alone is not sufficient to completely prevent the nuclear targeting of nucleoplasmin. Each cluster encodes a weak nuclear-targeting signal and together the two clusters generate a strong NLS. The mutational analysis of the NLS of CaM kinase is significant in that it clearly demonstrates that unlike the bipartite NLS, this single NLS is necessary and sufficient for its nuclear targeting.

Replacement of either of the first two lysine residues of the NLS of δ_B-CaM kinase with asparagine blocks nuclear targeting whereas only the first lysine in the SV40 T Ag NLS is essential. Changes in the other lysine residues of the SV40 T Ag NLS cause a partial defect in the nuclear targeting of the T Ag but do not completely block it. However, even 72 h after transfection, the Lys^{329Asn} mutant of α-(δ_B)-CaM kinase is still completely excluded from the nucleus, suggesting that the second lysine of the NLS is also essential (Fig. 7). It is possible that additional sequences in the SV40 T Ag stabilize the interaction of the NLS with the NLS-binding protein and therefore replacement of the lysine residues other than the first one may not disrupt the overall interaction sufficiently.

It is clear that the KKRK sequence, generated by the introduction of the 11-amino acid sequence by alternative splicing, is the NLS which targets δ_B-CaM kinase to the nucleus. The chimeric analysis further demonstrates that this NLS functions independently of any δ-CaM kinase specific sequences. We predict that other CaM kinase isoforms which encode a similar KKRK sequence would be targeted to the nucleus. Both α_B-CaM kinase and γA-CaM kinase encode an identical NLS generated by the introduction of an 11-amino acid and a 27-amino acid sequence, respectively, at a corresponding position (Fig. 6). Restricted PCR amplification of brain cDNA has identified a partial α-CaM kinase like cDNA clone containing a 33-bp insert (encoding 11 amino acids), likely inserted by alternative splicing (Benson et al., 1991). Indeed we find that α-CaM kinase containing the α_{33} PCR fragment which encodes for the 11-amino acid insert sequence (α_B-CaM kinase) is targeted to the nucleus (unpublished observations). Thus, the nuclear localization of α_B-CaM kinase further supports the role of the KKRK sequence in targeting CaM kinase to the nucleus.

Nuclear entry of CaM kinase may be modified by phosphorylation or autophosphorylation. The phosphorylation of serine residues near the NLS of a variety of nuclear-targeted proteins like SV40 T Ag, modifies their nuclear targeting (Rihs et al., 1991). The four serine residues immediately following the NLS sequence of δ_B-CaM kinase are candidates for regulatory phosphorylation. CaM kinase IV phosphorylates a peptide derived from γ_A-CaM kinase, that corresponds to the δ_B NLS-containing insert sequence (Miyano et al., 1992). Phosphorylation of these and other serine and threonine residues near the NLS of the nuclear CaM kinase isoforms could further modify the nuclear targeting of CaM kinase.

The targeting of the transfected δ_B-CaM kinase to the nucleus of cardiac myocytes suggests that even in the native environment of the cardiac cell, the NLS of δ_B-CaM kinase is available to the NLS-binding proteins and is not masked by an association of the kinase with some other cytosolic or cytoskeletal protein. Rat heart contains both δ_B- and δ_C-CaM kinase isoforms but neither the isoform composition of the holoenzymes nor their localization in myocytes is known (Edman and Schulman, 1994). Presumably the ratio of nuclear to non-nuclear subunits and the amount of putative-anchoring proteins in the cytoplasm/cytoskeleton will determine the distribution of the endogenous CaM kinase in heart.

Previous studies suggest that the α- and β-CaM kinase isoforms copurify and may therefore exist as a heteromultimer (for review see Rostas and Dunkley, 1992). Although a direct demonstration is still lacking, antibody specific to α-CaM kinase is reported to immunoprecipitate this isoform along with β-CaM kinase (Miller and Kennedy, 1985). However, EM analysis suggests that the enzyme consists of homomultimers of either α- or β-CaM kinase (Kanaseki et al., 1991). The potential for coassembly can be most easily tested by coexpressing identified isoforms and this approach has previously been used to demonstrate coassembly of the two neuronal isoforms (α- and β-CaM kinases) (Yamauchi et al., 1989). The present study uses the same approach to demonstrate the formation of heteromultimers composed of two non-neuronal isoforms of CaM kinase and of one neuronal and one non-neuronal isoform of CaM kinase. It appears that many cells and tissues express more than one isoform of CaM kinase and we show that several combinations of heteromultimers are possible. It will therefore be necessary to define which specific isoforms can coassemble before cellular targeting of CaM kinase can be understood.

The number of nuclear vs non-nuclear subunits of CaM kinase appear to determine the subcellular targeting of CaM kinase. For example, the gradual decrease of the transfected nuclear isoform as compared to the non-nuclear isoform is accompanied by a decrease in nuclear localization of both subunits and a concomitant increase in their cytosolic/cytoskeletal localization. In a cell, the isoform composition of the holoenzyme is probably dependent on the concentration of each subunit being synthesized and the nature of the as-

sociation domain which may place limits on which isoforms coassemble. The level of expression of the individual isoforms could be differentially regulated at the level of RNA transcription, translation, or processing. In addition, CaM kinase mRNA shows distinct localization in the brain. In particular, α-CaM kinase mRNA is present in dendrites but β-CaM kinase is not (Benson et al., 1992; Burgin et al., 1990). Thus, the cell body may contain heteromultimers, since mRNAs for both α- and β-CaM kinase isoforms are present there, whereas dendrites probably contain homomultimers of CaM kinase which contain only α-CaM kinase subunits. The potential interaction of multiple isoforms of CaM kinase introduces one more level at which the cellular localization of CaM kinase may be regulated and therefore introduces another mechanism for the regulation of its cellular functions.

We wish to thank Dr. H. Blau and Dr. R. Scheller for the use of their fluorescent microscopes.

This work was supported by National Institutes of Health grant GM 40600 and GM 30179. M. Srinivasan is a recipient of a postdoctoral fellowship from the Cystic Fibrosis Foundation.

Received for publication 31 March 1994 and in revised form 19 May 1994.

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