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Cloning and sequencing of multifunctional Ca^{2+} /calmodulin-dependent protein kinase from human lymphocytes

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protein kinase from human lymphocytes**

Saati, Shahin M., M.A.

San Jose State University, 1994

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**CLONING AND SEQUENCING OF MULTIFUNCTIONAL
Ca²⁺/CALMODULIN-DEPENDENT PROTEIN KINASE FROM HUMAN
LYMPHOCYTES**

A Thesis

Presented to

The Faculty of the Department of Biological Sciences
San Jose State University

In Partial Fulfillment
of the Requirements of the Degree
Master of Arts

By

Shahin M. Saati

May, 1994

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This Thesis is dedicated to great sufi master, Hazrat Salaheddin Ali Nader Shah Angha, the current leader and teacher of the Oveyssi school of sufism.

Words can not express my gratitude to him, for directing me to find the fortunate way of living, for caring, guiding, and teaching the positive way of being in all aspects of life. It was under his protection that I was able to start and finish my study.

ABSTRACT

CLONING AND SEQUENCING OF MULTIFUNCTIONAL Ca^{2+} /CALMODULIN-DEPENDENT PROTEIN KINASE FROM HUMAN LYMPHOCYTES

by Shahin M. Saati

The purpose of this study was to isolate and clone multifunctional Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase) from human lymphocytes. The cloning of human lymphocyte CaM kinase will facilitate future studies aimed at understanding details of the Ca^{2+} -dependent activation pathway of chloride channels in cystic fibrosis patients. This disease is due to a defect in the chloride channel, termed CFTR (cystic fibrosis transmembrane conductance regulator). CaM kinase has been shown to mediate the Ca^{2+} pathway that regulates non-CFTR chloride channels. This cloned CaM kinase gene will be helpful in future studies aimed at understanding its effect(s) in lymphocyte activation.

The present research has succeeded in cloning two isoforms of CaM kinase (termed γ_B and γ_C) from human lymphocytes. These are the first human isoforms of CaM kinase and the first clones from a tissue other than brain. Both clones resemble the γ isoform from rat brain. They differ from rat CaM kinase γ isoform in having two amino acid deletions, e.g., a 21 amino acid deletion at residue 316 and an 11 amino acid deletion at residue 356 (relative to γ isoform of rat brain CaM kinase). γ_B has an additional difference, manifested as an insertion of 23 amino acids at residue 356.

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I am also grateful to my friend, Paul Nghiem, for his guidance and encouragement throughout the course of this work and for teaching me the necessary skills for doing research.

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INTRODUCTION

Cystic Fibrosis (CF) is a lethal genetic disease characterized by defects in regulation of a chloride channel in the cell membrane by the cAMP-dependent protein kinase (PKA). The disease is due to a mutation in the gene product, termed Cystic Fibrosis Transmembrane conductance regulator (CFTR). One of the processes which has been of great interest in recent years is activation of chloride channels by increasing the level of intracellular free Ca^{2+} . In contrast to the cAMP-dependent pathway for chloride channel activation (which is defective), the Ca^{2+} pathway is functional in CF cells. The multifunctional Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase) has been shown to mediate the Ca^{2+} pathway that regulates non-CFTR chloride channels. Several isoforms of CaM kinase have been cloned from rat brain. Human isoforms and isoforms from tissues affected in CF, such as epithelial cells and lymphocytes, have not been cloned yet.

The purpose of this study was to isolate and clone the CaM kinase gene from human lymphocytes. This, in turn, will facilitate further studies on the localization and stability of this enzyme and its possible involvement in a variety of cellular processes. The cloning of human lymphocyte CaM kinase can also make possible future investigation into the Ca^{2+} -dependent activation pathway of chloride channels in CF patients. Site directed mutagenesis of the kinase can be used to make a constitutively active kinase that is independent of Ca^{2+} . This cloned lymphocyte gene thus makes possible numerous experimental approaches aimed at furthering our understanding of CaM kinase and its role in lymphocyte activation.

The techniques used in this work included highly degenerate primers (on the basis of conserved regions between different rat brain CaM kinases) to isolate isoforms from human lymphocytes, polymerase chain reaction (PCR) for amplifying the intended regions in lymphocytes, screening with use of labeled PCR product in order to identify or isolate the gene from the libraries, and anchored PCR for obtaining the 5' end of the cloned gene for sequence analysis.

My work was a collaboration with Dr. Phyllis Gardner of the Department of Medicine, Professor Howard Schulman of the Department of Pharmacology, Dr. Christine Martens of the Department of Immunology at the DNAX Research Institute, and their MD/PhD student, Paul Nghiem. These researchers had started working on the chloride channel activation pathway. This activation appears to be affected in the genetic disease, cystic fibrosis. Their work focused on the activation of chloride channels via increases in intracellular free calcium, a pathway which is preserved in cystic fibrosis. In contrast, the cAMP pathway for regulation of chloride conductance is defective in cystic fibrosis (Nishimoto et al., 1991; Chen et al., 1989).

When I started my work, they had already shown that multifunctional Ca^{2+} /calmodulin dependent protein kinase (CaM kinase) is involved in this pathway of channel activation in both epithelial cells and in lymphocytes. Early work on cloning the human lymphocyte CaM kinase had been started by Paul Nghiem, and it was the continuation of this project, along with Paul, which I chose to pursue.

CaM kinase from human lymphocyte would be the first human CaM kinase and also the first CaM kinase to be cloned from a tissue other than brain. At the time of Paul Nghiem's research, a partial clone of the lymphocyte CaM kinase gene had been isolated. DNA sequence spanning the entire length of the coding region would allow structural comparisons with neuronal forms, functional studies, gene over expression studies, mutational effects, and constitutive studies aimed at elucidating the role of CaM kinase in lymphocyte activation.

We cloned two different isoforms of this CaM kinase from human lymphocytes. One (γ_C) is actually a PCR product which was subcloned later into a vector. The other (γ_B) was isolated from a cDNA library. Both cloned forms of CaM kinase differ from the rat brain γ isoform in the variable region (Fig. 3).

LITERATURE REVIEW

The immune system functions to provide an organism with a flexible and active mechanism to respond specifically and non-specifically to a wide variety of antigens. Specific responses after recognition of antigen by antigen specific lymphocytes lead to a variety of cellular responses.

T and B lymphocytes represent the two antigen specific components of the cellular immune system. B-cells are transformed after stimulation with antigen into plasma cells, produce specific immunoglobulins or antibodies and mediate "humoral immunity." In contrast, T-cells participate in cell-mediated immune responses. After activation, T cells undergo complex changes, including cell differentiation and proliferation. As a result of clonal selection only a particular set of antigen specific T-lymphocytes is activated by a particular antigen, resulting in the clonal expansion of those particular antigen-reactive T-cells.

Activation of T-lymphocytes originates as a consequence of ligand-receptor interactions that occur at the interface of the T-cell and an antigen-presenting cell (APC), and involves stimulation of the T-cell antigen receptor (TCR). Antigen is the ligand for specific receptors expressed on specific clones of T-lymphocytes. Antigen typically exists as a peptide fragment, generated by proteolytic processing from a larger molecule. This antigen is then physically associated with a syngeneic major histocompatibility complex (MHC) antigen (Babbitt et al., 1985; Townsend et al., 1986) and presented to the Ag-receptor complex on the T-ymphocyte.

The antigen receptor complex on most T-cells is a multichain structure composed of a disulfide-linked α/β heterodimer (Ti) that is non-covalently associated with the three to seven invariant chains comprising the CD3 complex (Weiss and Imboden, 1987; Allison and Lanier, 1987). Antigen-induced stimulation of the TCR may be a primary stimulus in initiating activation. Other cell surface molecules expressed on resting T-cells (accessory molecules) may have roles in the process of cellular activation by: a) acting as adhesive molecules (making the interaction between the T-cell and APC more durable), b) changing the transmembrane signal initiated via the antigen receptor, and/or c) initiating their own signal transduction events, distinct from those of the TCR ligands, which have been called the alternative pathways of T-cell activation (Weiss and Imboden, 1987).

The interaction of the antigen receptor complex with processed antigen and MHC, leads to the initiation of cellular activation via transmembrane signaling. Such signal transduction is accompanied by the formation of intracellular biochemical mediators which have been referred to as "second messengers." These biochemical signals initiate or influence cellular response pathways. During T-cell activation, after the initiation of signal transduction, some responses (increases in cyclic nucleotides, production of cytoplasmic free calcium [Ca^{2+}], changes in pH, and protein phosphorylation) occur within minutes or hours, while others occur days after the stimulation event. Early cellular responses may arise directly or indirectly from TCR-mediated signal transduction. The later cellular responses, such as proliferation, usually are the result of a complex cascade of gene activation events and the subsequent influence of the products of these genes.

Changes in second messengers

T-cell activation is accompanied by changes in the level of several second messengers. Following stimulation of T-cells, cAMP levels initially rise and then fall. High levels of cAMP prevent T-cell proliferation (Wang et al., 1978).

Changes in pH can have effects on the activities of enzymes. Cellular alkalinization is known to occur during receptor-induced phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis in many cell types. The increase in pH following TCR stimulation may result from an increase in the activity of the plasma membrane Na⁺/H⁺ anti-porter subsequent to increased intracellular [Ca²⁺] and activation of Ca²⁺/calmodulin-dependent pathway(s) (Rosoff and Cantley, 1985; Grinstein et al., 1984).

The stimulation of (PIP₂), which is a relatively rare phospholipid in the membrane, causes the activation of an intracellular enzyme, phospholipase C. Phospholipase C hydrolyzes the phosphodiester linkage of PIP₂, resulting in the formation of inositol 1,4,5-trisphosphate (1,4,5-IP₃) and 1,2-diacylglycerol (DG). These intracellular "second messengers" induce an increase in [Ca²⁺] and protein kinase C (PKC) activation. PKC is a potent signal transduction system involved in transmembrane signaling by TCR (T-cell receptor) (within seconds following stimulation of TCR by antigen) (Nishizuka, 1986; Berridge and Irvine, 1984; Majerus et al., 1988).

1,4,5-IP₃ is a water soluble sugar with a specific intracellular receptor that regulates the mobilization of [Ca²⁺] from intracellular Ca²⁺ stores and across the cell membrane from outside (Gardner, 1989). Mobilization of intracellular Ca²⁺ is involved in the initial increase in [Ca²⁺] during the first 1 to 3 minutes following TCR stimulation (Imboden and Stobo, 1985). However, stimulation of the TCR induces increases in [Ca²⁺] which persist for several hours (Goldsmith and Wiess, 1988; Weiss and Imboden, 1987) and therefore requires a transmembrane flux from the outside of the cell to inside. Electrophysiologic studies have suggested that the mechanism responsible for the regulation of this transmembrane flux of Ca²⁺ in T cells involves a non-voltage-gated Ca²⁺ channel that can be regulated by 1,4,5-IP₃ (Kuno et al., 1986; Kuno and Gardner, 1987).

The other hydrolysis product of PIP₂ is diacylglycerol (DG). DG is also a potent "second messenger." It regulates a 78-kd Ca²⁺/phospholipid-dependent kinase, PKC (Fig. 1). Activation of PKC, which is a serine and threonine kinase, translocates the enzyme's activity from the cytosol to the plasma membrane (Nishizuka, 1986). It is likely that the increase in [Ca²⁺] which follows inositol phosphate generation can also lead to the activation of calcium/calmodulin-activated kinases.

Protein phosphorylation

A large number of cytoplasmic and membrane proteins become phosphorylated on serine, threonine, and tyrosine residues within seconds to minutes following TCR stimulation (Shackelford and Trowbridge, 1986; Patel et al., 1987). While protein phosphorylation has attracted great interest, few of the phosphorylated substrates have been characterized.

Calmodulin

Calmodulin, a Ca^{2+} -binding protein found in all animal and plant cells examined to date, can constitute as much as 1% of the total protein mass of the cell. Calmodulin serves many functions within the cell and is encoded by a single polypeptide chain of about 150 amino acid residues. This sequence includes four high affinity Ca^{2+} -binding sites. Calmodulin undergoes a large conformational change when it binds to Ca^{2+} . Raising the free Ca^{2+} level (often via phosphatidylinositol signaling system and stimulation of TCR), activates a variety of processes, including changes in the conformation of Ca^{2+} -binding calmodulin. Ca^{2+} -calmodulin complexes activate specific target proteins, including Ca^{2+} /calmodulin-dependent protein kinases.

Each type of cell has a characteristic set of target proteins that are regulated by calmodulin, enabling the cell to make its own distinctive response to a change in intracellular Ca^{2+} level. Through the signaling cascade mediated by Ca^{2+} the responses to

extracellular signals can be greatly amplified (as well as regulated) by a variety of inputs.

FIG. 1: Comparison between cAMP-dependent protein kinase, PKA; protein kinase C, PKC; and CaM kinase. Three kinds of serine and threonine kinases that can phosphorylate substances in the presence of second messengers.

CaM kinase is a member of a family of Ca^{2+} /calmodulin-dependent protein kinases which also includes Ca^{2+} /calmodulin-dependent protein kinases I and III, myosin light chain kinase and phosphorylase kinase (reviewed in Colbran, 1989). This kinase is also called by other names, including calmodulin-dependent glycogen kinase, type II Ca^{2+} /calmodulin-dependent protein kinase, Ca^{2+} /calmodulin-dependent protein kinase II and calmodulin-dependent multiprotein kinase (reviewed in Schulman, 1991).

In brain this multimeric enzyme (CAM) consists of 10-12 subunits. Two subunit isoforms are primarily in neuronal tissues, the α (54 kd) and β (60 kd) isoforms. Two isoforms are widely distributed, the γ (59 kd) and δ (60 kd) isoforms. Each of these isoforms is represented by a distinct gene except the β' -subunit (58 kd), which is an alternatively spliced form of the β isoform (Fig. 2) (reviewed in Schulman, 1991).

This enzyme isolated from certain regions of brain comprises about 1% of total protein (Erondy and Kennedy, 1985) and is a mixture of different ratios of α , β and β' subunits. The forebrain enzyme is relatively enriched for the α subunit and the cerebellum for the β subunit (Hanson et al., 1989). CaM kinase is present in both membrane and cytosolic fractions in each tissue examined (Schulman and Greengard, 1978; Hashimoto and Soderling, 1987). Isoforms have been identified in a variety of tissues, including liver (Payne et al., 1983), skeletal muscle (Woodgett et al., 1983), and heart (Schulman and Greengard, 1978).

Each of these subunits consists of a protein kinase catalytic domain in the N-terminal half of the molecule which is followed by a regulatory domain consisting of a calmodulin binding site and an autoinhibitory domain (Fig. 2). This part is followed by an association domain that is responsible for subunit-subunit interaction and possibly for association with the cytoskeleton and other cellular structures. This general organization is seen in two other second messenger regulated kinases, cAMP kinase and protein kinase C (Fig. 1).

There is a great deal of homology between the five subunit isoforms of CaM kinase (α , β , β' , γ , δ). Differences occur mostly in the variable region where the regulatory and the association domains join (Fig. 2). In these areas, small segments of DNA are inserted or deleted. These segments may be important for the expression of specific functions (i.e. regulatory interactions, kinetics of transcription, and developmental activation) unique to particular kinases (Bulliet et al., 1988, reviewed in Schulman, 1991).

CaM kinase autophosphorylates itself (as well as other cell proteins) when it is activated by Ca^{2+} -calmodulin complexes. It is kept inactive in its basal state by the putative autoinhibitory subdomain whose function is disrupted by binding of Ca^{2+} /calmodulin, leading to disinhibition of the kinase (Fig. 2) (reviewed in Schulman, 1991). The resulting autophosphorylation causes the enzyme to become independent of Ca^{2+} , thereby prolonging the duration of kinase activity beyond the duration of the initial activating Ca^{2+} signal. The Ca^{2+} -independent autophosphorylation will maintain the active state until cellular phosphatases remove the phosphate and deactivate it.

FIG. 2: Comparison between five different isoforms of CaM kinase in rat. The α isoform has no insertion or deletion. The β isoform has two insertions, of 24 and 39 amino acids. β' is like β except it has a deletion of 15 amino acids. γ has two insertions of 21 and 26 amino acids, and δ has two insertions of 34 and 21 amino acids. All these deletions/insertions are in the variable region between the calmodulin binding and association domains.

Studies on the activation of this enzyme have shown that there are several serine and threonine residues in the "hinge" region of the kinase (between the catalytic and calmodulin-binding domains) which are actively autophosphorylated (Lin et al., 1987). It has been shown that phosphorylation of Thr²⁸⁶ is essential for converting the kinase to a Ca²⁺-independent form (Hanson et al., 1989).

CaM kinase has a broad substrate specificity. This kinase has been shown to phosphorylate enzymes involved in neurotransmitter synthesis and carbohydrate metabolism as well as structural proteins, such as the cytoskeletal proteins involved in neurotransmitter release and in microtubule function (reviewed in Schulman, 1988). Recently it has been shown that a constitutive form of CaM kinase is capable of increasing the transcription rate of specific genes independent of protein kinase C activation (Kapiloff, and et al., 1991), while studies on CaM kinase have pointed to its involvement in mitosis (Ohta et al., 1990).

FIG. 3: Comparison between CaM kinase γ isoform from rat (γ_A) with γ_B and γ_C , from human lymphocytes. γ_C has two deletions, of 21 and 11 amino acids; and γ_B has these two deletions of 21 and 11 amino acids along with an insertion of 23 amino acids. All these variations are also in the variable region.

METHODS

This project was highly collaborative in nature, and frequently involved Paul Nghiem (PN) and myself (SS). At the heading of each method the primary person(s) involved in that phase are indicated.

cDNA Synthesis: PN and SS

Total RNA was isolated from human jurkat T cells using guanidinium thiocyanate lysis followed by CsCl centrifugation (Chirgwin et al., 1979; Sambrook et al., 1989). One microliter of total RNA served as template for cDNA synthesis by Avian Myoblastosis Virus (AMV) reverse transcriptase (Boehringer Mannheim, Indianapolis, IN) primed with oligo (dT) (Krung et al., 1987). AMV reverse transcriptase (200 U/ μ l) was diluted 1:10 in dilution buffer (10% glycerol, 10 mM potassium phosphate, pH 7.4, 0.2% Triton X-100, 2 mM dithiothreitol) and was left on ice with occasional vortexing for 30 minutes, and 2 μ l of the diluted enzyme was used for each reaction. RNA was heated to 65°C for 10 minutes. Then the reaction mix was assembled in the following order: H₂O to a final volume of 20 μ l, 10x AMV RT buffer [0.5 M Tris-HCl pH 8.3, 0.1 M MgCl₂, 0.5 M KCl, 10 mM DTT, 100 mM EDTA, 100 mg/ml BSA] 2 μ l, oligo (dT) 100 μ g/ml (or RT primer 10 OD/ml) 1 μ l, dNTP mixture (10 mM each) 2 μ l, spermidine-HCL (10 mM) 1 μ l, RNasin (Promega) 1 μ l, sodium pyrophosphate (80 mM) 1 μ l, template RNA < 1 μ g, and diluted AMV RTase 2 μ l. The reaction was then incubated at 42°C for 45-60 minutes. cDNA was used immediately or frozen at -20°C.

PCR amplification reactions: PN and SS

PCR amplification was performed essentially by the method of Sambrook (Sambrook et al., 1989). Taq polymerase and 10X Taq polymerase buffer were supplied by IBI (New Haven, CT). Ultrapure nucleotides were obtained from Pharmacia (Piscataway, NJ): dA, dG, dC, and dT were each used at a final concentration of 125 μ M (stock solution was 10x). Primers (were prepared by PN, see table 1) were diluted in water to 10 OD/ml (approximately 25 pmole/ μ l). Reaction mixture for amplification: water to 50 μ l, 10x Taq polymerase buffer 5 μ l, dNTP mixture 5 μ l, primers 1 μ l of each, cDNA 1 μ l, and enzyme 0.2 μ l. Tubes were overlaid with 2 drops of paraffin oil, spun down briefly and were cycled as specified.

Purification and direct sequencing of PCR products: PN and SS

PCR products were run on a 1.2% agarose gel in order to compare their sizes to the predicted size. In some cases, amplified products were sequenced directly to verify their identities. In order to obtain optimal double stranded-DNA sequencing template, PCR products were gel-purified, electroeluted (Sambrook et al., 1989), phenol-choloroform extracted, precipitated with ethanol and dried. Amplified products were sequenced directly to verify their identities. In other cases, asymmetric PCR was used to produce single stranded DNA: about 1% of an agarose gel-purified 50 μ l reaction was used as template for a second amplification with 0.5 pmole of limiting primer and 5.0 pmole of excess primer in a 100 μ l reaction volume (Erich, 1989). After ethanol precipitation, the products were directly used for sequencing.

Use of highly degenerate primers to amplify and sequence cDNA: PN

Highly degenerate primers [CK1.S (4096-fold degenerate) and CK1.A (1024-fold degenerate), based on conserved regions of the known rat brain CaM kinases, were used to isolate the human lymphocyte form of the same kinase.

Total RNA was isolated and used as a template for reverse transcription primed with oligo(dT). The cDNA from this reaction was subjected to amplification. The PCR protocol consisted of 40 cycles of: 1 minute at 94°C, 1.5 minutes at 46°C, 2 minutes at 72°C. The final cycle was then followed by a single 10 minute extension at 72°C. The relatively low annealing temperature was used to optimize hybridization of degenerate primers to specific sequences with minimum cross-hybridization to unrelated sequences. The long extension time was used to allow amplification of the full cDNA (1.0 kb cDNA) (Nghiem, 1990).

Subcloning of PCR products into pUC 18: PN

PCR products were digested with Sal I (all restriction enzymes were obtained from IBI, New Haven, CT)) as follows: 16.5 µl of H₂O, 5 µl (about 0.5 µg) of DNA, 2.5 µl of 10x buffer, and Sal I 1.0 µl (8 units), and left at 37°C for 5 hours. Following digestion, 8 µl 5 M NH₄OAC pH 7.5 (for elimination of cut ends of Sal I primers which can recombine with vector) and 100 µl EtOH were added and the tubes were left on dry ice for 15 minutes. After centrifuging for 10 minutes, the DNA was washed with 70% EtOH and resuspended in 20 µl TE buffer. Vector (pUC 18, from IBI) was also cut with Sal I, treated with calf intestinal phosphatase, and the fragments were ligated as follows: 0.5 µl pUC 18 (about 20 ng) and 5.0 µl insert (100 ng), were mixed with 0.5 µl ATP (100 mM), 0.5 µl DTT (0.2 M), 1.0 µl 10x ligase buffer (200 mM Tris pH 7.6, 50 mM MgCl₂, 50 mM dithiothreitol, 500 µg/ml BSA), 1.5 µl H₂O, and 1.0 µl ligase (4 unit/µl). The mixtures were ligated at 15°C overnight.

Base denaturation sequencing and sequence analysis of double-stranded DNA: PN and SS. The later researcher was primarily responsible for sequencing and analyzing both the cDNA library truncated clone g_B, and the subcloned PCR product g_C.

DNA sequencing was carried out using dideoxynucleotide termination (Sanger et al., 1977) and Sequenase reagents (U. S. Biochemical Corp.) on double stranded DNA that had been denatured with NaOH according to the manufacturer's specifications. Base denaturation of double-stranded DNA has proven to be a reliable and simple approach for DNA sequencing. Either 50 µl of a PCR reaction (gel-purified, electroeluted, phenol-chloroform extracted, precipitated with ethanol and dried) or 2 µg of cesium chloride purified plasmid DNA were resuspended in 9 µl of water, 1 µl 2M NaOH/ 2 mM EDTA was added, and the mixture was left at room temperature for 5 minutes for denaturation. The sequencing primer was added (approximately 5 pmole) prior to neutralization with 3 µl of 3 M sodium acetate, pH 5.2. The addition of the primer prior to neutralization allowed the elimination of the annealing step. After ethanol precipitation and centrifugation, the dried DNA was resuspended in 8 µl water and 2 µl 5x sequencing buffer from the sequenase version 2.0 DNA sequencing kit. This mixture was warmed to 37°C for 5 minutes, and the standard Sequenase protocol was followed from the labeling step. Sequencing reactions were run on standard urea, 6%-8% acrylamide sequencing gels of 0.25 mm thickness. Sequences were entered into the GCG (Devereux, Haeber, and Smithies, 1984) sequence analysis program software package version 7.0, April 1990, on the Stanford University Cell Biology Vax computer facility.

Oligolabeling the DNA probe with ^{32}P : PN and SS

An oligolabeling kit (Pharmacia, Piscataway, NJ), was used for carrying out the labeling step essentially as described by Feinberg and Vogelstein (1984), labeling 25-50 ng of DNA to $1\text{--}2 \times 10^9$ dpm/ μg . DNA was dissolved in TE buffer at a concentration such that 25-50 ng can be added to the reaction in a volume of no more than 34 μl . The DNA was denatured by heating for 2-3 minutes in a water bath at 95-100°C. The DNA was immediately placed on ice for > 2 minutes, then the tube was centrifuged briefly. The following were added to a clean microcentrifuge tube: denatured DNA (25-50 ng) 20 μl ; reagent mix [containing: dNTP's, random hexamer ss DNAs, and buffer (buffered aqueous solution containing dATP, dGTP, dTTP, and random hexadeoxyribonucleotides)] 10 μl ; [$\alpha\text{-}^{32}\text{P}$]dCTP (3000 Ci/mmol) 5 μl ; distilled water 14 μl ; Klenow fragment 1 μl , and the mixture was incubated at 37°C for 30-60 minutes. ^{32}P probe was then separated from unincorporated ^{32}P dCTP by a G-25 column, Boehringer Mannheim (Indianapolis, IN), in the following manner. The top and then bottom were taken off and the column was placed on the receptacle tube. The buffer was allowed to drain, then it was forced out using positive pressure with a pasteur pipet bulb. The tubes were then wrapped with parafilm and centrifuged at (3000 RPM, 2000 g) for 2 minutes. The receptacle with the buffer was discarded and a new receptacle was used, wrapped carefully with parafilm. The probe mixture was poured directly onto the center of the gel column (not to run down the sides, bypassing the gel) and was centrifuged at 3300 RPM for 4 minutes. A geiger counter "quick test" of the relative amount of label in probe versus in the column was used. The radiolabeled probe was generally at least 2 times more radioactive than the column, which contained unincorporated label.

Plasmid library Screening: PN and SS.

Plasmid library screening was carried out essentially as described in Sambrook (1989). Bacteria were plated directly from a transformation mixture onto detergent-free nitrocellulose filters; replica filters were prepared by filter-to-filter contact. The dry nitrocellulose filters were numbered with an indelible marking pen. Using sterile, blunt-ended forceps, a sterile filter was laid, numbered side down, on a day-old agar plate containing the appropriate antibiotics. Then the filter was peeled off, inverted, and placed on a plate, numbered side up. The bacteria were applied in a small volume of liquid (<0.4 ml, containing up to 10,000 bacteria for an 82-mm filter). Then the liquid was spread over the surface of the filter with a sterile, bent glass rod. A border 2-3 mm wide was left at the edge of the filter free of bacteria. The plates stood at room temperature until all of the liquid had been absorbed. The plates were inverted and incubated at 37°C until very small colonies (0.1-mm diameter) appeared (about 8-10 hours). Replicas were prepared by filter-to-filter contact and master plates were sealed and stored well labeled at 4°C. After growing colonies further (to about 1 mm in diameter), nitrocellulose filters were treated with: 1) 10% SDS, 3 minutes; 2) 0.5 M NaOH-1.5 M NaCl, 5 minutes; 3) 0.5 M Tris, pH 7.6, 1.5 M NaCl, 5 minutes; and 4) 2x SSC, 5 minutes. They were U.V. cross-linked (Stratalinker, Stratagene) and then transferred to a glass crystallizing dish containing 200 ml of prewashing solution (5x SSC, 0.5% SDS, 1 mM EDTA pH 8.0) at 50°C. The filters were stacked on top of one another in the solution. The dish was covered with Saran Wrap and transferred to a rotating platform in an incubator. In all subsequent steps, the filters were slowly agitated to prevent them from sticking to one another. The filters were incubated for 30 minutes at 50°C. The solution was changed, and the bacterial debris was gently scraped from the surfaces of the filters using a Kimwipe soaked in prewashing solution. This reduced background hybridization due to bacterial lipids and proteins without diminishing

the positive signal. The filters were transferred to 50 ml of prehybridization solution [10% SDS, 500 mM sodium pyrophosphate, in Modified Denhardt's solution (1% ficoll, 1% polyvinylpyrrolidone, 1% gelatin, in 6x SSC, and autoclaved) plus 0.5 ml of 10 mg/ml salmon sperm DNA (boiled and cooled on ice for 5 minutes) in a glass crystalizing dish. The filters were incubated for 1 hour at 68°C. 25 ml of prehybridization solution plus 0.25 ml of salmon sperm DNA (boiled and cooled on ice) and 100 ng of ³²P-labeled DNA probe (boiled and cooled on ice) were used for hybridization. During the hybridization, which was carried out for 6-18 hours, the container holding the filters was tightly closed to prevent the loss of fluid by evaporation. When the hybridization was complete, the hybridization solution was removed and the filters were immediately immersed in a large volume (300-500 ml) of 2x SSC and 0.1% SDS at room temperature. The filters were gently agitated and at least once during washing they were turned over. After 5 minutes, the filters were transferred to a fresh batch of wash solution and agitated gently. The washing procedure was repeated twice more. At no stage during the washing procedure were the filters allowed to dry. The filters were washed twice for 1-1.5 hours in about 50 ml of a solution of 1x SSC and 0.1% SDS at 68°C. This was repeated until very little probe came off during the wash. Then the moist filters were placed between two sheets of Saran Wrap and exposed to film. Positive colonies were picked up from master plates and the process was essentially repeated for subsequent screenings.

Plasmid miniprep: PN and SS

A 2 ml overnight culture (*E. coli* DH5a) with antibiotic selection (Amp 50 µg/ml) was grown and 1.5 ml were transferred to a microfuge tube, spun for 1 minute, and the supernatant was discarded. Cells were resuspended in 0.35 ml STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA pH 8.0, 50 mM Tris pH 8.0), 10 µl of lysozyme solution (30 mg/ml in 10 mM Tris pH 8.0), vortexed briefly, and placed in a boiling water bath for 3 minutes. Then they were spun down for 15 minutes at room temperature. The pellet was taken out with a toothpick and discarded. To the supernatant, 330 µl isopropanol was added and after 15 minutes, the supernatant was spun at 4°C. The pellet was rinsed with 70% ethanol, air dried, and resuspended in 25 µl TE plus 20 µg/ml DNase-free RNase A and incubated at 65°C for 15 minutes to dissolve the pellet completely. DNA was stored at -20°C.

Preparation of frozen competent *E. coli*: PN and SS

A 5 ml overnight culture of *E. coli* DH5a was grown to stationary phase. DH5a is a trademark of BRL (Gaithersburg, MD). It is *recA1*, is readily transformed by large plasmids, no endonuclease 1, which improves quality of plasmid DNA made from minipreps. 5 ml were inoculated into 500 ml LB in a fernbach flask, and grown with shaking for approximately 2-3 hours at 37°C to O.D. approximately 0.8-0.6 (600 nm). Then the culture was collected into 50 ml polypropylene centrifuge tubes, and chilled on ice for 10-15 minutes. The cells were pelleted at 1000 g (2-3,000 rpm in clinical centrifuge at 4°C for 12-15 minutes) and the medium was drained completely. Pellets were resuspended by moderate vortexing in a volume of Buffer RF1 (RbCl 100 mM, MnCl₂ 50 mM, K-acetate 30 mM, CaCl₂ 10 mM, Glycerol 15%, pH was adjusted to 5.8 with 0.2 N acetic acid and sterilized by filtration through a prerinsed 0.22 µm membrane). This was 1/4-1/3 of the volume collected (e.g. 125-167 ml). The cells were incubated on ice for 20-30 minutes, spun again, then resuspended in buffer RF2 (MOPS 10 mM, RbCl 10 mM, CaCl₂·2H₂O 75 mM, Glycerol 15%; pH was adjusted to 6.8 with NaOH and sterilized by filtration through a prerinsed 0.22 µ membrane) to 1/15 of original volume (e.g. 30 ml), distributed into chilled screw cap tubes or microfuge tubes, frozen immediately in a dry ice/EtOH bath, and then placed at -70°C.

Use of low melting agarose for isolation of DNA fragments to be ligated: PN and SS

A 1% low melting agarose gel in T.A. buffer (no EDTA) was poured. Relatively large amounts of appropriately digested vector (pCDL-Not1-SR α) and insert were run (about 2 μ g). The bands were cut out using a hand-held long wavelength U.V. light (to avoid inducing U.V. damage to the DNA). These gel slices were melted at 65°C. 9 μ l of insert and 1 μ l of vector slices (melted) were placed in a tube and after remelting (1 minute at 65°C), 10 μ l of a ligation mixture were added [4 μ l 5x ligase buffer (100 mM Tris-Cl pH 7.6, 25 mM MgCl₂, 25 mM dithiothreitol, 250 μ g/ml BSA), 5 μ l H₂O, 1 μ l T4 DNA ligase (4 unit/ μ l)]. After placing at 14°C overnight, they were transformed as described below.

Transformation of frozen competent cells: PN and SS

Frozen competent cells were thawed on ice. For each transformation, 100 μ l of cell suspension were aliquoted into a 12x 75 mm sterile polystyrene or polypropylene tube on ice. Then 1 ml of the ligation mixture of 1-10 ng of intact plasmid was added. They were left on ice for 10 to 30 minutes. They were then shocked in a 42°C water bath for 1-2 minutes. Then 1 ml of LB broth was added and the tubes were left at 37°C for 30-60 minutes. The cells were spun down at 4000 rpm for 10 minutes, resuspended in 0.5 ml, plated on LB plus ampicillin (50 μ g/ml) plates, and were left at 37°C overnight.

Large scale plasmid prep: PN

After growing overnight, with the appropriate antibiotic, *E.coli* containing the desired plasmid were spun down at 3000 rpm for 15 minutes. The supernatant was poured off and the pellet was resuspended thoroughly in 25 ml solution I (50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA, filtered and stored at 4°C) and transferred to 250 ml Nalgene bottles. 50 ml of solution II (0.2 N NaOH, 1% SDS) were added and swirled gently. The mixture was left on ice for 10 minutes and then 37.5 ml of solution III (3 M potassium acetate, 11.5% glacial acetic acid) were added. The bottle was inverted until the two phases became one and left on ice for 10 minutes. The bottle was spun at 8000 rpm for 15 minutes, the supernatant was decanted into a clean 250 ml Nalgene bottle, and 120 ml of isopropanol was added and left for 20 minutes at room temperature. The bottle was spun at 8000 rpm for 10 minutes and the pellet was dried and dissolved in 5 ml of TE. This mixture was transferred to a 50 ml polypropylene tube and 4 ml of 1:1 phenol:chloroform (100 mM Tris pH 8 saturated) was added and vigorously vortexed. The tube was spun for 5 minutes at 3000 rpm and the supernatant was transferred to a clean 30 ml glass tube. 15 ml EtOH were added and mixed and the solution was left at room temperature for 20 minutes and spun for 10 minutes at 8000 rpm. The supernatant was removed and the pellet was washed with 70% EtOH, resuspended with 3 ml TE, and 4.3 g CsCl were added to the mixture. Then 200 µl EtBr (10 mg/ml) were added. By using a 3 ml syringe and an 18 gauge needle, samples were loaded into Beckman Quik-seal tubes(1/2x2") and sealed using a heat sealer. Tubes were placed into a rotor and were spun overnight at 70,000 rpm, 20°C. The plasmid DNA band was pulled out by using an 18 gauge needle. The DNA was transferred to a new tube, filled with CsCl/TE/EtBr solution (1.1 g CsCl per 1 ml TE to which EtBr 50 µl per ml was added) and sealed and spun at 70,000 rpm overnight at 20°C. The upper band of nicked and genomic DNA was removed with a 1 ml syringe and an 18

gauge needle and discarded. Then the lower band of plasmid DNA was pulled out by an 18 gauge needle. It was placed in a 15 ml tube and 1 ml TE/NaCl-saturated isopropanol was added to the tube. After vortexing, the upper phase (isopropanol) was removed to the EtBr waste container. This was repeated until no pink came out and then twice more. Then 4 ml TE was added and mixed. 10 ml EtOH was added and after 15 minutes at room temperature, the tube was centrifuged at 8000 rpm (4°C) for 15 minutes. The pellet was washed with 70% EtOH and resuspended in 0.4 ml TE. This was transferred to a 1.5 ml screw-cap tube. 50 µl 3M NaOAc 1 ml EtOH were added and mixed and after sitting at room temperature for 15 minutes, it was spun in an Eppendorf centrifuge. The DNA was washed with 70% EtOH, dried briefly and resuspended in 300-500 µl TE. An O.D. was taken to determine the concentration and an aliquot of the DNA was digested and run on a gel to check the quality.

Anchored PCR: PN

Anchored PCR was carried out essentially as described by Frohman et al. (1988) and by Loh et al. (1989). These two techniques differ in that the Frohman approach uses dATP for tailing the cDNA, while the Loh approach requires dGTP. Both approaches were carried out simultaneously through the first round of anchored PCR and in this case only, the Frohman approach yielded the desired product.

Total RNA from Jurkat T cells was reverse transcribed as described (see Methods) with either a gene specific primer (CKG 90.A) or oligo (dT). The quality of the initial cDNA was assessed by verifying that a target sequence of CaM kinase could be amplified. The cDNA mixture was purified of enzymes and dNTPs by repeated washings on a Centricon 100 (Amicon) microconcentrator. Tailing was carried out as described (Frohman) with terminal deoxynucleotide transferase (USB) for 1 hour at 37°C. The tailed cDNA was then amplified using several combinations of primers, varied annealing temperatures and lengths of extension time(s) for amplification. The conditions which proved successful were 10 pmoles oligo (dT)-adaptor plus 25 pmoles Race.outer (both served as sense primers) and 25 pmoles CK102.A, which were used to amplify cDNA which had been primed with oligo (dT) in the following protocol: 1 cycle of: 94°C, 3 minutes; 46°C, 2 minutes; 72°C, 10 minutes; followed by 30 cycles of 94°C, 1 minute; 55°C, 1 minute; and 72°C, 2 minutes.

PCR products were run on 1.2% agarose, blotted and probed with ³²P end-labeled oligomers (CKG 60.S and CKG 57.A) specific for CaM kinase and located upstream (5')

of the specific antisense primers used for amplification. A faint smear of specific hybridization showed up in the correct location (approximately 250-400 base pairs) in the lane generated with these conditions. The successful product from this first round of anchored PCR was used in a second, "nested" round of PCR. The conditions were: 25 pmole each of Race.inner (sense primer), CKG 90.A (antisense), and 1 μ l of the previous PCR reaction, using the exact same amplification protocol as the first round. These PCR products were run on both agarose (1.2%), and acrylamide (6%) gels, blotted and hybridized as above and found to contain substantial amounts of the predicted product.

An internal Sal I site known to be present in γ_B CaM kinase toward the 5' end (at nucleotide # 196 relative to the translational start site), within the region targeted by anchored PCR, was used to verify the identity of this PCR product. The DNA was cut with Sal I, run on a gel and blotted. After digestion, the smear-like band ran at the predicted size of about 75 base pairs shorter than the undigested DNA.

This PCR product was subcloned into Bluescript II KS+ (Stratagene), by digestion with Sal I and Cla I [a Cla I site was built into the oligo (dT)-adaptor for anchored PCR] and a total of 12 of the more than 100 colonies obtained were sequenced. Of these 12 clones, 11 were independent (one appeared twice) and 2 were full length (extended beyond the 5' translational start site), both of which contained a distinct point mutation. In one of these full length clones, however, this mutation was in a "wobble" position, still encoding the same amino acid. All anchored PCR clones were derived from γ_B CaM kinase, as judged by the identity of 41 nucleotides which overlapped between the cDNA library clone and all 11 independent anchored PCR clones, as well as by homology to the rat brain γ_A isoform. These anchored PCR clones allowed determination of 22 nucleotides of the 5'

non-coding portion of the mRNA, which contained a Kozak consensus translational initiation sequence (Kozak, 1989). This insert, corresponding to the 5' portion of γ_B CaM kinase, was isolated by digestion with Sal I and Cla I and ligated into Bluescript in a trimolecular ligation in which the vector was digested with Not I /Cla I, treated with calf intestinal phosphatase, and the 3' fragment of human γ_B CaM kinase was digested with Sal I/Not I. This procedure yielded a full length human γ_B CaM kinase, the first 196 bases of which were derived from an anchored PCR product and the remaining 3' fragment was derived from the plasmid library clone (Fig. 8, Fig. 9).

RESULTS

We reasoned that the human isoforms of CaM kinase would be homologous to the ubiquitous (γ and δ) isoforms cloned from rat brain, and therefore utilized PCR to attempt to clone the human lymphocyte isoform(s). Total RNA, isolated from a human T-lymphocyte cell line (Jurkat), was used as a template for reverse transcription primed with oligo (dT). After cDNA had been made, it was amplified for 40 cycles of amplification using highly degenerate primers (CK1.S and CK1.A) (see Methods). Products were run on a 1.2% agarose gel and appeared to be the predicted size. Then they were sequenced using asymmetric PCR (see Methods). About 150 base pairs were readable, enough to prove that the product was a CaM kinase isoform.

This cDNA was used for another amplification reaction using the CK1.S primer (close to N-terminal) and COOH.A (close to C-terminal). These two primers were designed to be as close to the ends of the predicted gene product as possible, while falling within a region which was still highly conserved between the previously described CaM kinase isoforms. The PCR products were run on a gel and were of the predicted size of about 1100 base pairs.

In order to be able to subclone these PCR products, they were subjected to another amplification reaction with primers possessing Sal I sites (CK1.Sal.S and COOH.Sal.A) and were gel purified. The pUC 18 was cut with Sal I and used as a vector for subcloning the PCR product.

TABLE 1: Primers for sequencing γ_B and γ_C isoforms of human lymphocyte CaM kinase

Name (S=Sense) (A=Anti)	Length	3' end of primer corresponds to γ_B Nucleotide #	Isoform-like	Degeneracy	Restriction sites	Sequence (5' TO 3')
COOH.Sal.A	30-mer	1290	γ and δ	64-fold	Sal I	GCCGTCGACAAAGTA(A or G) AA(C or T)(C or T)(A or G)TG (A or G)AA(A or G)TC
CK1.Sal.S	30-mer	85	γ and δ	8-fold	Sal I	CCGGTCGACITTTGCGGCCGCT TGG(A or G or C or T)AA(A or G)GG
CK1.S	25-mer	85	γ and δ	4096-fold		TTTGGCGCCGCTTGG(A or T or G or C)AA(G or A)GG(A or T or G or C)GC(A or T or G or C)TT(T or C)(A or T)(G or C)(A or T or G or C)GT
CK1.A	25-mer	837	γ and δ	1024-fold		TTTCCGCGGTT(A or T or G or C)AG(A or T or G or C)GT (T or C)TC(T or C)TG(A or G or C or T)C(G or T)(A or G)TGCAT
MLATA	21-mer	922	α	non		GAAGTTCCTGGCGGCCAGCAT
EDEA	21-mer	931	α	non		GTCCTCATCCCTGATGGTGGT
CBS.S	27-mer	927	γ and δ	32-fold		AAGGG(A or T)GCCATC(C or T)(C or G)AC(A or C)AC(C or T)ATGCT

Name (S=Sense) (A=Anti)	Length	3' end of primer corresponds to γB Nucleotide #	Isoform-like	Degeneracy	Restriction sites	Sequence (5' TO 3')
COOH.A	27-mer	1290	γ and δ	64-fold		AAAGTA(A or G)AA(C or T)(C or T)T(A or G)TG(A or G)AA(A or G)TCCATCCC
CAMK-inner	24-mer	304	δ	non		CTGGAGGTGAAC TGT TGAAT ACAT
SRA.A	28-mer	-	-	non		TTATCATGTCTGGATCAGCIT GGGCTGC
SRA.S	32-mer	-	-	non		CTAGGCCTGTACGGAAAGTGT ACTTCGTCTCT
CKGV.A	26-mer	948	γB	non		TGTGGCTCCTTGACGCCGCC ATCTG
CKG134.S	25-mer	399	γ	non		CCACATCCACCAGCATGACAT CGTC
CKG385.S	23-mer	1126	γ	non		GGGCTCCACAGAGAGCTGCA ACA
CKG375.S	17-mer	1097	γ	non		CATAATGCTACAGATGG
CK213.A	22-mer	637	γ and δ	2-fold		GCTGATC(C or T)TCATCCCA GAAGGG
CK259.S	20-mer	773	γ and δ	4-fold		ATG(C or T)TGACCAT(A or G)AACCCTGC

Name (S=Sense) (A=Anti)	Length	3' end of primer corresponds to γB Nucleotide #	Isoform-like	Degeneracy	Restriction sites	Sequence (5' TO 3')
CK278.A	17-mer	838	γ and δ	8-fold		TG(C or G)C(G or T)(A or G)TG CATCATGGA
CK102.A	23-mer	298	γ and δ	8-fold		TA(C or T)TCTCT(G or T)GCC AC(A or T)ATGTCCTC
CKG485.S	17-mer	1424	γ	non		GCCTACATCCGGGCTCAC
CKG90.A	17-mer	261	γ	non		CAAGGTCAAAACACGAGG
CKG60.A	17-mer	212	γ	non		CGACTTCTGAAACATCC
SRA.A1	17-mer	-	-	non		CTGCATTCTAGTTGTGG
CKG430.S	17-mer	1253	γ	non		TGTGATCCAGGCCCTCAC
CKG57.A	17-mer	160	γ	non		TCTAGTTTCTGGTGATC
RQET.S	23-mer	866	α	512-fold		ATGCA(C or T)(A or C)G(A or G or C or T)CA(A or G)GA(A or G)AC(A or G or C or T)GT(A or G)GA

After transformation using *E. Coli* MC1061 frozen competent cells, colonies were grown overnight for 24 hours on LB plus ampicillin (100 µg/ml) plates, then plasmid minipreps were prepared. DNA was cut with Hind III and was run on a 1.2% agarose gel in order to identify a plasmid with the insert. One clone contained the 1.0 kb PCR product insert and was subsequently named " γ_C CaM kinase." At this stage a large scale plasmid preparation was carried out to have plenty of this clone for sequencing and for use as a probe for library screening.

Sequencing strategy for γ_C

Primers used for sequencing (for primer locations see table 1) included: CBS.S, RQET.S, MLAT.A, EDE.A, CK278.A, CK GV.A, CK 259.S, CK 213.A, CK INNER.S and CK 134.S, and utilized a numbering system corresponding to the full length of γ_B clone (Fig. 9). As shown in figure 4, these primers revealed sequences between nucleotides #: 947-1119, 957-988, 729-895, 829-1027, 509-765, 738-907, 854-1119, 357-590, 357-592, and 441-602. We found that the beginning of the clone (base pair # 196 to # 357, Fig. 5) was unknown. While the bases spanning # 357-592 were known. The region spanning #592-602 was also unknown, base pair # 602-1119 was known, and base pair # 1119 until the end of the clone was unknown. We next utilized primers including: CK 134.S, CKG375.S and M13⁻ (reverse on orientation) for the region between base pair # 592-602 and the 3' and the 5' ends of the clone, respectively. Electrophoresis times were extended in order to resolve regions farther from the primers. When sequencing of this clone was completed, we found the clone highly similar to the γ -like isoform of rat brain CaM kinase. On the basis of homology between them, however, it appeared to be truncated

at both the 3' and 5' ends, missing 196 base pairs at the 5' end and 438 base pairs at the 3' end (Fig. 5). This γ_C clone extended from base pair # 196 to base pair # 1119.

Fig. 4: Sequencing strategy for γ_C . Schematic showing all the primers used as well as regions sequenced with each primer.

At this stage we began screening a Jurkat T cell cDNA library (a gift from Naoko Arai, DNAX Research Institute). Jurkat cDNA was ligated into the pCDL- NOT I- SR α vector in BamH I and Not I sites. The titer determined was 10^9 /ml (100 μ l of that would give about 10^8 colonies) with a complexity of about 10^5 independent colonies. This vector is a standard pBR322-based plasmid which is ampicillin resistant and has the SV40 early promotor and RU-5 HTLV I LTR (Takebe et al., 1988) which allows this DNA to be very efficiently transcribed and translated in eukaryotes, facilitating expression screening and high expression of foreign genes for various applications.

Fig. 5: Nucleotide sequence and deduced amino acid sequence of the γ_C polypeptide of CaM kinase II from human lymphocytes. Nucleotides are numbered so that they correspond to the numbering system of γ_B .

196	CGA	CTT	CTG	AAA	CAT	CCA	AAC	ATC	GTG	CGC	CTC	CAT	GAC	AGT	ATT	TCT	GAA	GAA	GGG	TTT	255
66	Arg	Leu	Leu	Lys	His	Pro	Asn	Ile	Val	Arg	Leu	His	Asp	Ser	Ile	Ser	Glu	Glu	Gly	Phe	85
	R	L	L	K	H	P	N	I	V	R	L	H	D	S	I	S	E	E	G	F	
256	CAC	TAC	CTC	GTG	TTT	GAC	CTT	GTT	ACG	GGA	GGG	GAG	CTG	TTT	GAA	GAC	ATC	GTG	GCC	AGA	315
86	His	Tyr	Leu	Val	Phe	Asp	Leu	Val	Thr	Gly	Gly	Glu	Leu	Phe	Glu	Asp	Ile	Val	Ala	Arg	105
	H	Y	L	V	F	D	L	V	T	G	G	E	L	F	E	D	I	V	A	R	
316	GAG	TAC	TAC	AGT	GAA	GCT	GAT	GCC	AGC	CAC	TGT	ATA	CAT	CAG	ATT	CTG	GAG	AGT	GTT	AAC	375
106	Glu	Tyr	Tyr	Ser	Glu	Ala	Asp	Ala	Ser	His	Cys	Ile	His	Gln	Ile	Leu	Glu	Ser	Val	Asn	125
	E	Y	Y	S	E	A	D	A	S	H	C	I	H	Q	I	L	E	S	V	N	
376	CAC	ATC	CAC	CAG	CAT	GAC	ATC	GTC	CAC	AGG	GAC	CTG	AAG	CCT	GAG	AAC	CTG	CTG	CTG	GCG	435
126	His	Ile	His	Gln	His	Asp	Ile	Val	His	Arg	Asp	Leu	Lys	Pro	Glu	Asn	Leu	Leu	Leu	Ala	145
	H	I	H	Q	H	D	I	V	H	R	D	L	K	P	E	N	L	L	L	A	
436	AGT	AAA	TGC	AAG	GGT	GCC	GCC	GTC	AAG	CTG	GCT	GAT	TTT	GGC	CTA	GCC	ATC	GAA	GTA	CAG	495
146	Ser	Lys	Cys	Lys	Gly	Ala	Ala	Val	Lys	Leu	Ala	Asp	Phe	Gly	Leu	Ala	Ile	Glu	Val	Gln	165
	S	K	C	K	G	A	A	V	K	L	A	D	F	G	L	A	I	E	V	Q	
496	GGA	GAG	CAG	CAG	GCT	TGG	TTT	GGT	TTT	GCT	GGC	ACC	CCA	GGT	TAC	TTG	TCC	CCT	GAG	GTC	555
166	Gly	Glu	Gln	Gln	Ala	Trp	Phe	Gly	Phe	Ala	Gly	Thr	Pro	Gly	Tyr	Leu	Ser	Pro	Glu	Val	185
	G	E	Q	Q	A	W	F	G	F	A	G	T	P	G	Y	L	S	P	E	V	
556	TTG	AGG	AAA	GAT	CCC	TAT	GGA	AAA	CCT	GTG	GAT	ATC	TGG	GCC	TGC	GGG	GTC	ATC	CTG	TAT	615
186	Leu	Arg	Lys	Asp	Pro	Tyr	Gly	Lys	Pro	Val	Asp	Ile	Trp	Ala	Cys	Gly	Val	Ile	Leu	Tyr	205
	L	R	K	D	P	Y	G	K	P	V	D	I	W	A	C	G	V	I	L	Y	
616	ATC	CTC	CTG	GTG	GGC	TAC	CCT	CCC	TTC	TGG	GAT	GAG	GAT	CAG	CAC	AAG	CTG	TAT	CAG	CAG	675
206	Ile	Leu	Leu	Val	Gly	Tyr	Pro	Pro	Phe	Trp	Asp	Glu	Asp	Gln	His	Lys	Leu	Tyr	Gln	Gln	225
	I	L	L	V	G	Y	P	P	F	W	D	E	D	Q	H	K	L	Y	Q	Q	
676	ATC	AAG	GCT	GGA	ACC	TAT	GAT	TTC	CCA	TCA	CCA	GAA	TGG	GAC	ACG	GTA	ACT	CCT	GAA	GCC	735
226	Ile	Lys	Ala	Gly	Thr	Tyr	Asp	Phe	Pro	Ser	Pro	Glu	Trp	Asp	Thr	Val	Thr	Pro	Glu	Ala	245
	I	K	A	G	T	Y	D	F	P	S	P	E	W	D	T	V	T	P	E	A	
736	AAG	AAC	TTG	ATC	AAC	CAG	ATG	CTG	ACC	ATA	AAC	CCA	GCA	AAG	CGC	ATC	ACG	GCT	GAC	CAG	795
246	Lys	Asn	Leu	Ile	Asn	Gln	Met	Leu	Thr	Ile	Asn	Pro	Ala	Lys	Arg	Ile	Thr	Ala	Asp	Gln	265
	K	N	L	I	N	Q	M	L	T	I	N	P	A	K	R	I	T	A	D	Q	
796	GCT	CTC	AAG	CAC	CCG	TGG	GTC	TGT	CAA	CGA	TCC	ACG	GTG	GCA	TCC	ATG	ATG	CAT	CGT	CAG	855
266	Ala	Leu	Lys	His	Pro	Trp	Val	Cys	Gln	Arg	Ser	Thr	Val	Ala	Ser	Met	Met	His	Arg	Gln	285
	A	L	K	H	P	W	V	C	Q	R	S	T	V	A	S	M	M	H	R	Q	
856	GAG	ACT	GTG	GAG	TGT	TTG	CGC	AAG	TTC	AAT	GCC	CGG	AGA	AAA	CTG	AAG	GGT	GCC	ATC	CTC	915
286	Glu	Thr	Val	Glu	Cys	Leu	Arg	Lys	Phe	Asn	Ala	Arg	Arg	Lys	Leu	Lys	Gly	Ala	Ile	Leu	305
	E	T	V	E	C	L	R	K	F	N	A	R	R	K	L	K	G	A	I	L	
916	ACG	ACC	ATG	CTT	GTC	TCC	AGG	AAC	TTC	TCA	GCT	GCC	AAA	AGC	CTA	TTG	AAC	AAG	AAG	TCG	975
306	Thr	Thr	Met	Leu	Val	Ser	Arg	Asn	Phe	Ser	Ala	Ala	Lys	Ser	Leu	Leu	Asn	Lys	Lys	Ser	325
	T	T	M	L	V	S	R	N	F	S	A	A	K	S	L	L	N	K	K	S	
976	GAT	GGC	GGT	GTC	AAG	GAG	CCA	CAA	ACC	ACT	GTG	GTA	CAC	AAC	GCT	ACA	GAT	GGG	ATC	AAG	1035
326	Asp	Gly	Gly	Val	Lys	Glu	Pro	Gln	Thr	Thr	Val	Val	His	Asn	Ala	Thr	Asp	Gly	Ile	Lys	345
	D	G	G	V	K	E	P	Q	T	T	V	V	H	N	A	T	D	G	I	K	

1036	GGC TCC ACA GAG AGC TGC AAC ACC ACC ACA GAA GAT GAG GAC CTC AAA GTG CGA AAA CAG	1095
346	Gly Ser Thr Glu Ser Cys Asn Thr Thr Thr Glu Asp Glu Asp Leu Lys Val Arg Lys Gln	365
	G S T E S C N T T T E D E D L K V R K Q	
1096	GAG ATC ATT AAG ATT ACA GAA CAG C	1119
366	Glu Ile Ile Lys Ile Thr Glu Gln	373
	E I I K I T E Q	

The plasmid library was screened using the subcloned PCR product (human γ_C CaM kinase) that had been labeled to high ^{32}P specific activity (see Methods). In the first screening only one colony was positive. This region was picked from the master plate and an aliquot of a solution containing colonies from this region was plated. In the second screening, 100 positive colonies were obtained. Colonies were picked from the master plates, plated in two dilutions and grown overnight. In the third screening the positive colonies were picked up and plated in three dilutions. At each stage the density of colonies on the plates was diluted as much as possible while still having at least one positive colony. On the fourth screening, a single positive well isolated colony was picked. From this colony, which was diluted and plated, every new colony was positive. Plasmid miniprep DNA was prepared and cut with BamH I and Not I and run on a gel. The results showed two DNA fragments, one 3300 bp (vector) and the other 2200 bp (CaM kinase insert). Southern blot analysis showed that the probe hybridized to the insert of this clone (which was later designated human γ_B CaM kinase). A large scale plasmid prep was prepared in order to sequence human γ_B CaM kinase.

Sequencing strategy for γ_B

Sequencing clone γ_B , using primers CK213.A, CK278.A, CKGV.A, RQET.S, CBS.S, CKG3752.A, CKG385.S, CKG430.A, CKG485.S. resulted in sequence information from base pair # 508-610, 651-844, 749-931, 920-988, 943-1175, 1152-1234, 1251-1478 and 1555-1670, respectively (using the convention that nucleotide number 1 was the translational start site). This information, however, still provided no sequence data between the start codon and base pair # 508, from base pair # 610-651, and from base pair # 1670 until the end of the clone. We next employed the primers CK213.A, CKG485.S, CK278.A, and SR α .A1, and electrophoresed them for various times in order to resolve regions farther from the primers. These results revealed the entire sequence of the whole clone through the stop codon at the 3' end, although we did not yet have the sequence of the 5' end of the clone (Fig. 6). We next used the primers CKG57.A and CKG90.A to give the sequence beyond base pair # 264 at the 5' end, and employed the primers SR α .S and SR α .A1 to find the boundaries of kinase and vector. In the end we were able to sequence from the 5' end of this clone through its entire length to the 3' poly A tail and junction with SR α (Fig. 7, Fig. 9).

Comparison of the human isoform with those from rat brain showed the human clone was truncated 158 base pairs from the 5' end, although it was complete beyond the stop codon at the 3' end (Fig. 7). However, ligated onto the 5' end of the clone was approximately 600 bases of DNA sequence that did not correspond to CaM kinase, SR α or any sequence in the GeneBank. This was taken to be a cloning artifact and was not analyzed further. Unfortunately, screening the cDNA library further did not yield a longer

full length clone at the 5' end. Paul, Therefore, carried out an anchored PCR to obtain the 5' portion of the clone.

Figure 6: Sequencing strategy for γ_B . Schematic showing all primers used as well as regions sequenced with each primer. Different lengths of running time are represented by slight variations in names of sequencing runs with the same primer (e.g., CK2131.A, CK2132.A, CK2133.A).

FIG. 7: Truncated γ_B CaM kinase in pCDL-Not1-SRalpha. Demonstrating cloning artifact at the 5' end of the clone upstream of the intrinsic Sal I site. This clone was isolated from a Jurkat cDNA plasmid library.

FIG. 8: γ_B in SRalpha. Demonstrating ligation at Sal I site of the 3' end of the anchored PCR product and 5' end of the truncated γ_B , cDNA library clone.

Figure 9: Nucleotide sequence and deduced amino acid sequence of the γ_B polypeptide of CaM kinase II from human lymphocytes. Nucleotides are numbered beginning with the translational start site obtained from anchored PCR. The 5' end non coding region conforms to the Kozak consensus sequence (Kozak, 1989).

	-22	TGC ACG CCG GTC CGC AGC	
1	ATG GCC ACC ACC GCC ACC TGC ACC CGT TTC ACC GAC GAC TAC CAG CTC TTC GAG GAG CTT	60	
1	Met Ala Thr Thr Ala Thr Cys Thr Arg Phe Thr Asp Asp Tyr Glu Leu Phe Glu Glu Leu	20	
M	A T T A T C T R F T D D Y Q L F E E L		
61	GGC AAG GGT GCC TTC TCT GTG GTC CGC AGG TGT GTG AAG AAA ACC TCC ACG CAG GAG TAC	120	
21	Gly Lys Gly Ala Phe Ser Val Val Arg Arg Cys Val Lys Lys Thr Ser Thr Glu Glu Tyr	40	
G	K G A F S V V R R C V K K T S T Q E Y		
121	GCA GCA AAA ATC ATC AAT ACC AAG AAG TTG TCT GCC CGG GAT CAC CAG AAA CTA GAA CGT	180	
41	Ala Ala Lys Ile Ile Asn Thr Lys Lys Leu Ser Ala Arg Asp His Glu Lys Leu Glu Arg	60	
A	A K I I N T K K L S A R D H Q K L E R		
181	GAG GCT CGG ATA TGT CGA CTT CTG AAA CAT CCA AAC ATC GTG CGC CTC CAT GAC AGT ATT	240	
61	Glu Ala Arg Ile Cys Arg Leu Leu Lys His Pro Asn Ile Val Arg Leu His Asp Ser Ile	80	
E	A R I C R L L K H P N I V R L H D S I		
241	TCT GAA GAA GGG TTT CAC TAC CTC GTG TTT GAC CTT GTT ACG GGA GGG GAG CTG TTT GAA	300	
81	Ser Glu Glu Gly Phe His Tyr Leu Val Phe Asp Leu Val Thr Gly Gly Glu Leu Phe Glu	100	
S	E E G F H Y L V F D L V T G G E L F E		
301	GAC ATT GTG GCC AGA GAG TAC TAC AGT GAA GCA GAT GCC AGC CAC TGT ATA CAT CAG ATT	360	
101	Asp Ile Val Ala Arg Glu Tyr Tyr Ser Glu Ala Asp Ala Ser His Cys Ile His Glu Ile	120	
D	I V A R E Y Y S E A D A S H C I H Q I		
361	CTG GAG AGT GTT AAC CAC ATC CAC CAG CAT GAC ATC GTC CAC AGG GAC CTG AAG CCT GAG	420	
121	Leu Glu Ser Val Asn His Ile His Glu His Asp Ile Val His Arg Asp Leu Lys Pro Glu	140	
L	E S V N H I H Q H D I V H R D L K P E		
421	AAC CTG CTG CTG GCG AGT AAA TGC AAG GGT GCC GCC GTC AAG CTG GCT TTT GGC CTA	480	
141	Asn Leu Leu Leu Ala Ser Lys Cys Lys Gly Ala Ala Val Lys Leu Ala Asp Phe Gly Leu	160	
N	L L L A S K C K G A A V K L A D F G L		
481	GCC ATC GAA GTA CAG GGA GAG CAG CAG GCT TGG TTT GCT GGC ACC CCA GGT TAC	540	
161	Ala Ile Glu Val Glu Gly Glu Glu Glu Ala Trp Phe Gly Phe Ala Gly Thr Pro Gly Tyr	180	
A	I E V Q G E Q Q A W F G F A G T P G Y		
541	TTG TCC CCT GAG GTC TTG AGG AAA GAT CCC TAT GGA AAA CCT GTG GAT ATC TGG GCC TGC	600	
181	Leu Ser Pro Glu Val Leu Arg Lys Asp Pro Tyr Gly Lys Pro Val Asp Ile Trp Ala Cys	200	
L	S P E V L R K D P Y G K P V D I W A C		
601	GGG GTC ATC CTG TAT ATC CTC CTG GTG GGC TAT CCT CCC TTC TGG GAT GAG GAT CAG CAC	660	
201	Gly Val Ile Leu Tyr Ile Leu Leu Val Gly Tyr Pro Pro Phe Trp Asp Glu Asp Glu His	220	
G	V I L Y I L L V G Y P P F W D E D Q H		
661	AAG CTG TAT CAG CAG ATC AAG GCT GGA GCC TAT GAT TTC CCA TCA CCA GAA TGG GAC ACG	720	
221	Lys Leu Tyr Glu Glu Ile Lys Ala Gly Ala Tyr Asp Phe Pro Ser Pro Glu Trp Asp Thr	240	
K	L Y Q Q I K A G A Y D F P S P E W D T		
721	GTA ACT CCT GAA GCC AAG AAG TTG ATC AAC CAG ATG CTG ACC ATA AAC CCA GCA AAG CGC	780	
241	Val Thr Pro Glu Ala Lys Asn Leu Ile Asn Glu Met Leu Thr Ile Asn Pro Ala Lys Arg	260	
V	T P E A K N L I N Q M L T I N P A K R		
781	ATC ACG GCT GAC CAG GCT CTC AAG CAC CCG TGG GTC TGT CAA CGA TCC ACG GTG GCA TCC	840	
261	Ile Thr Ala Asp Glu Ala Leu Lys His Pro Trp Val Cys Glu Arg Ser Thr Val Ala Ser	280	
I	T A D Q A L K H P W V C Q R S T V A S		
841	ATG ATG CAT CGT CAG GAG ACT GTG GAG TGT TTG CGC AAG TTC AAT GCC CGG AGA AAA CTG	900	
281	Met Met His Arg Glu Glu Thr Val Glu Cys Leu Arg Lys Phe Asn Ala Arg Arg Lys Leu	300	
M	M H R Q E T V E C L R K F N A R R K L		

901 AAG GGT GCC ATC CTC ACG ACC ATG CTT GTC TCC AGG AAC TTC TCA GCT GCC AAA AGC CTA 960
 301 Lys Gly Ala Ile Leu Thr Thr Met Leu Val Ser Arg Asn Phe Ser Ala Ala Lys Ser Leu 320
 K G A I L T T M L V S R N F S A A K S L

 961 TTG AAC AAG AAG TCG GAT GGC GGT GTC AAG CCA CAG AGC AAC AAC AAA AAC AGT CTC GTA 1020
 321 Leu Asn Lys Lys Ser Asp Gly Gly Val Lys Pro Gln Ser Asn Asn Lys Asn Ser Leu Val 340
 L N K K S D G G V K P Q S N N K N S L V

 1021 AGC CCA GCC CAA GAG CCC GCC CCC TTG CAG ACG GCC ATG GAG CCA CAA ACC ACT GTG GTA 1080
 341 Ser Pro Ala Gln Glu Pro Ala Pro Leu Gln Thr Ala Met Glu Pro Gln Thr Thr Val Val 360
 S P A Q E P A P L Q T A M E P Q T T V V

 1081 CAC AAC GCT ACA GAT GGG ATC AAG GGC TCC ACA GAG AGC TGC AAC ACC ACC ACA GAA GAT 1140
 361 His Asn Ala Thr Asp Gly Ile Lys Gly Ser Thr Glu Ser Cys Asn Thr Thr Thr Glu Asp 380
 H N A T D G I K G S T E S C N T T T E D

 1141 GAG GAC CTC AAA GTG CGA AAA CAG GAG ATC ATT AAG ATT ACA GAA CAG CTG ATT GAA GCC 1200
 381 Glu Asp Leu Lys Val Arg Lys Gln Glu Ile Ile Lys Ile Thr Glu Gln Leu Ile Glu Ala 400
 E D L K V R K Q E I I K I T E Q L I E A

 1201 ATC AAC AAT GGG GAC TTT GAG GCC TAC ACG AAG ATT TGT GAT CCA GGC CTC ACT TCC TTT 1260
 401 Ile Asn Asn Gly Asp Phe Glu Ala Tyr Thr Lys Ile Cys Asp Pro Gly Leu Thr Ser Phe 420
 I N N G D F E A Y T K I C D P G L T S F

 1261 GAG CCT GAG GCC CTT GGT AAC CTC GTG GAG GGG ATG GAT TTC CAT AAG TTT TAC TTT GAG 1320
 421 Glu Pro Glu Ala Leu Gly Asn Leu Val Glu Gly Met Asp Phe His Lys Phe Tyr Phe Glu 40
 E P E A L G N L V E G M D F H K F Y F E

 1321 AAT CTC CTG TCC AAG AAC AGC AAG CCT ATC CAT ACC ACC ATC CTA AAC CCA CAC GTC CAC 1380
 441 Asn Leu Leu Ser Lys Asn Ser Lys Pro Ile His Thr Thr Ile Leu Asn Pro His Val His 460
 N L L S K N S K P I H T T I L N P H V H

 1381 GTG ATT GGG GAG GAC GCA GCG TGC ATC GCC TAC ATC CGG CTC ACC CAG TAC ATC GAC GGG 1440
 461 Val Ile Gly Glu Asp Ala Ala Cys Ile Ala Tyr Ile Arg Leu Thr Gln Tyr Ile Asp Gly 480
 V I G E D A A C I A Y I R L T Q Y I D G

 1441 CAG GGT CGG CCT CGC ACC AGC CAG TCA GAA GAG ACC CGG GTC TGG CAC CGT CGG GAT GGC 1500
 481 Gln Gly Arg Pro Arg Thr Ser Gln Ser Glu Glu Thr Arg Val Trp His Arg Arg Asp Gly 500
 Q G R P R T S Q S E E T R V W H R R D G

 1501 AAG TGG CTC AAT GTC CAC TAT CAC TGC TCA GGG GCC CCT GCC GCA CCG CTG CAG TGA GCT 1560
 501 Lys Trp Leu Asn Val His Tyr His Cys Ser Gly Ala Pro Ala Ala Pro Leu Gln End 520
 K W L N V H Y H C S G A P A A P L Q *

 1561 CAG CCA CAG GGG CTT TAG GAG ATT CCA GCC GGA GGT TCA ACC TTC GCA GCC AGT GGC TCT 1620
 1621 GGA GGG CCT GAG TGA CAG CGG CAG TCC TGT TTG TTT GAG GTT TAA AAC AAT TCA ATT ACA 1680
 1681 AAA GCG GCA GCA GCC AAT GCA CGC CCC TGC ATG CAG CCC TCC CGC CCG CCC TTC GTG TCT 1740
 1741 GTC TCT GCT GTA CTG AGG TGT TTT TTA CAT TTA AAA AAA AAA AAA A

[illegible]

FIG. 10: Alignment of the deduced amino acid sequences of the variable regions of γ_B and γ_C CaM kinases from the human lymphocyte with α , β , β' , γ_A , and δ CaM kinases from rat. Dashes indicate no corresponding amino acid exists in that isoform.

DISCUSSION

CaM kinase is an enzyme with different isoforms. Rat brain CaM kinase is composed of several subunits with three isoforms α , β , and β' , in different proportions. There are two other isoforms, γ and δ , which are found in a variety of tissues. All of the isoforms are encoded by distinct genes, except β' which is the product of alternative splicing of the β transcript (reviewed in Schulman, 1991). Relative to the α subunit, β has two insertions, one of 24 amino acids at residue 316 and one of 39 amino acids at residue 356 (Fig. 10). β' is identical to β , except for a deletion of 15 amino acids (residues 380 to 394). The δ subunit has two insertions, one of 34 amino acids at residue 316 and one of 21 amino acids at residue 545. The γ subunit also has two insertions, one of 21 amino acids at residue 316 and the second of 26 amino acids at residue 545. All these differences are in the variable region, between the carboxy terminal and the calmodulin binding domain.

Our results indicate that we have cloned two isoforms of CaM kinase from human lymphocytes, γ_B and γ_C . These are the first isoforms of CaM kinase cloned from human tissue and also the first from a tissue other than brain. Both clones resemble the γ isoform from the rat. They differ from rat CaM kinase γ isoform in having two deletions, of 21 amino acids at residue 316 and 11 amino acids at residue 356 (331 of γ_B). γ_B has an additional difference which is an insertion of 23 amino acids at residue 356 (331 of γ_B). All of these changes are in the variable region as was the case with the rat CaM kinase isoforms (Fig. 10).

Alignment of the two new isoforms with the rat brain CaM kinase isoforms provide useful information about the functional regions of the enzyme. γ_B and γ_C have an ATP-binding sequence with a Gly-X-X-Ser (where X is any amino acid) sequence in amino acid residues 21-26 (Fig. 9) (Bulliet et al., 1988; Lin et al., 1987), a basic amino acid rich calmodulin-binding sequence, Arg-Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-Val, in residues 297-310 and a consensus sequences for phosphorylation by CaM kinase, Arg-X-X-Ser/Thr at residues 284-287 and 312-315. The consensus sequence for CaM binding contains Val only in γ subunits (while Ala is present in α , β , β' , δ). The catalytic site, Val-His-Arg-Asp-Leu-Lys-Pro-Glu-Asn-Leu-Leu-Leu, located in the N-region (amino acid residues 133-144), has been reported to be shared by most protein kinases (Bairoch, and Claverie, 1988; Brenner, 1987) and is found in these CaM kinase isoforms as well.

Residues RQET, which are important in producing an autonomous CaM kinase by autophosphorylation at threonine²⁸⁶ in the rat brain isoforms, are present in these two new isoforms. Both γ_B and γ_C contain a threonine within the consensus autophosphorylation site, RQET region. The fact that the threonine residue is conserved may support the previous observation that it is critical for autophosphorylation and enzyme regulation (Hanson et al., 1989).

The carboxy terminals of these two subunits is not homologous to the other known subunits. Thus, they may be important for possible unique functions of the CaM kinase in lymphocytes. Previous studies have shown that the carboxy terminal may function as an association domain. This domain is important in production of the holoenzyme and may be critical in interactions with organelles (Hanson et al., 1989). Therefore, differences in the structure of this domain may lead to differences in function among the isoforms. For

example, the forebrain enzyme is richer in α subunit and is likely to be included in postsynaptic densities, while cerebellar holoenzyme is richer in β subunits (Miller and Kennedy, 1985).

Recent observations demonstrate that truncation of the association domain of the α isoform changes targeting of the enzyme from cytoskeleton to nucleus (Hanson, unpublished results). Therefore, γ_B and γ_C , with differences in their association domain may differ in intracellular targeting. The association domain may contain targeting sequences which localize the enzyme to specific sites in the cell, hence affecting subsequent function. It is possible that other isoforms also exist. The position of deletions and insertions suggests that the isoforms may differ in the size of the oligomer, e.g., liver enzyme may be a hexamer, whereas the brain enzyme is a dodecamer.

Another possible function of the carboxy terminal may be its involvement in developmental control of isoform expression. For example, the β isoform can be expressed in early development and the α subunit can be synthesized during the maturation of neurons (Sayhoun et al., 1985). As γ_B and γ_C have deletions in their association domains, they may be expressed in different developmental stages.

Another possible aspect of deletions in the association domain has to do with control of the catalytic activity of CaM kinase. Since calmodulin activates the kinase by binding to the inhibitory domain and displacing it away from the catalytic site, differences in the variable region, which is located right after the calmodulin-binding domain, may affect calmodulin binding capacity and therefore the sensitivity of the enzyme to stimulation.

Comparison of γ_B and γ_C isoforms demonstrate that there are six nucleotides in γ_C which differ from γ_B . One of these mutations causes amino acid residue 230 to change from alanine to threonine in γ_C . The other mutations are silent and therefore do not cause any amino acid changes relative to γ_A or γ_B . Consensus splicing signals between the exon-intron intersections (Mount, 1982) are not present in γ_B and γ_C isoforms. These differences suggest that γ_B and γ_C may be derived from different transcripts. There is also the possibility that these nucleotide differences are PCR artifacts. Additional studies will be needed to determine whether γ_B and γ_C are alternatively spliced products of the same gene or whether they are derived from distinct genes.

Recent studies on γ_B show that it is readily expressed in COS cells (Nghiem, 1993). Analysis of its dependency on Ca^{2+} and functional studies should follow soon. The research demonstrates that since γ_B is active, it is likely that the splicing was done correctly. Future studies involving site directed mutagenesis would be helpful in generating a constitutively active kinase independent of Ca^{2+} . Studies in which the expression of the kinase is blocked (perhaps by the use of antisense oligomers) may also be helpful in understanding the role of CaM kinase in the cell (Nghiem, 1993).

Another aspect of future studies would involve T-lymphocyte activation. After stimulation of T-cells with antigen, the level of cAMP decreases in order to facilitate the proliferation of the stimulated cells. After stimulation, the activity of CaM kinase would presumably increase via the phosphatidyl inositol pathway. This may be important in keeping the cell in the active state in the absence of cAMP.

Another possible direction for study on these isoforms would be their potential participation in the production of "memory" in lymphocytes. During stimulation, the Ca^{2+} deficient enzyme is exposed to Ca^{2+} . Due to its autoregulation characteristic, this exposure would convert it to a Ca^{2+} independent form. This conversion may be important in producing a memory stage or keeping the cell in the stimulated state when there is no longer any stimulation to activate the enzyme.

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