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Cloning and Analysis of Two New Isoforms of Multifunctional Ca^{2+}/Calmodulin-dependent Protein Kinase

EXPRESSION IN MULTIPLE HUMAN TISSUES*

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Multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) is a mediator of calcium signals in diverse signaling pathways. In human lymphocytes and epithelial tissues, CaM kinase activates a chloride channel via a Ca^{2+}-dependent pathway which is preserved in cystic fibrosis. To characterize the CaM kinase present in these tissues we have cloned an isoform of this kinase from human T lymphocytes. We show the cDNA structure of two variants of this human CaM kinase, γ_B and γ_C, which are predicted to translate to 518 and 495 amino acids, respectively. Amino acid differences between these isoforms and the rat brain γ isoform (which we refer to as γ_A) are localized to the variable domain. We used RNase protection of this variable region to reveal the level of expression of γ_B and γ_C CaM kinase mRNAs in nine human tissues and cell lines. When transfected into Jurkat T cells, the γ_B cDNA encoded a functional kinase which cosedimented on sucrose gradients with endogenous T cell CaM kinase activity and formed a large multimeric enzyme. The recombinant γ_B isoform displayed two phases of autophosphorylation characteristic of CaM kinases, including the phase which converts it to a partially Ca^{2+}-independent species. Site-directed mutagenesis of the predicted autoinhibitory domain yielded a mutant which was ~37% active in the absence of Ca^{2+}/calmodulin, confirming the region as critical for autoregulation, and suggesting this mutant as a tool for studying the role of CaM kinase in nonneuronal tissues.

Multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase)[1] is a ubiquitous enzyme mediating diverse effects of hormones and neurotransmitters that utilize Ca^{2+} as a second messenger (1, 2). CaM kinase is present in most tissues as an oligomer, composed of 6–12 subunits, depending on the isoform and tissue. Although CaM kinase has been purified from several mammalian tissues, relatively little is known about the structure of CaM kinase isoforms outside of the nervous system. Differences between the cloned mammalian isoforms consist mostly of 11–39-amino acid insertions and deletions in the variable region which lies between the calmodulin binding domain and the association domain. Thus far, five isoforms (α, β, β', γ, and δ) have been cloned from mammalian (rat) brain. Expression of the α and β isoforms of CaM kinase appears to be mostly confined to the brain. The rat brain γ and δ isoforms appear to be more widespread as RNA blot analysis shows transcription in many rat tissues (3).

All isoforms share a highly conserved catalytic domain at the amino-terminal portion of the molecule, an autoinhibitory sequence overlapping with a calmodulin binding region, followed by an association domain which is important in holoenzyme formation. Immunoelectron microscopy of CaM kinase purified from rat brain indicates that the kinase has an overall "flower"-like structure, with a central core composed of 8–10 association domains from which radiate globular catalytic domains (4). This study also suggests that the α isoform may assemble into a decamer, while β forms an octamer.

Studies of regulation of the kinase via autophosphorylation have revealed an intriguing mechanism by which CaM kinase can maintain activity following a transient rise in the free Ca^{2+} concentration (reviewed in Ref. 5). As Ca^{2+} increases, Ca^{2+}-calmodulin binds to and activates CaM kinase, causing the kinase to phosphorylate itself in the autoinhibitory domain (on Thr^{286}) and leading to two interesting effects on its activity. First, autophosphorylation on Thr^{286} sharply decreases the rate of dissociation of calmodulin such that even after free Ca^{2+} levels diminish, calmodulin is trapped, transiently fixing the kinase at maximal Ca^{2+}-stimulated activity (6). Second, after dissociation of calmodulin, the presence of a phosphate at Thr^{286} disrupts the autoinhibitory domain and results in a kinase with Ca^{2+}-independent activity of 20–80% of maximal Ca^{2+}-stimulated activity, depending on the substrate and conditions of assay (7–10). This second property formed the basis of an approach to generate a Ca^{2+}-independent or constitutive mutant of CaM kinase by mimicking autophosphorylation at Thr^{286} by changing this residue to an aspartic acid. This mutant had ~20–40% activity in the absence of Ca^{2+}/calmodulin stimulation, could be activated to 100% by Ca^{2+}/calmodulin and, when microinjected into *Xenopus* oocytes, induced initiation of maturation in the absence of a Ca^{2+} stimulus (11, 12).

A role for CaM kinase has been identified in some nonneuronal tissues, including regulation of a chloride-specific ion

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[1] The abbreviations used are: CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; CF, cystic fibrosis; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid; kb, kilobase(s).

channel in human tissues, with potential significance for the disease cystic fibrosis (CF) (13–15). The most common lethal genetic disease in Caucasians, CF leads to defective regulation of chloride ion transport in a variety of tissues causing death by compromising the function of secretory epithelia of the lung and gut. A major pathway for activation of chloride conductances via cAMP-dependent protein kinase is blocked by mutations in the CF gene, the cystic fibrosis transmembrane conductance regulator. A parallel pathway utilizing Ca^{2+} as a second messenger remains functional in CF and has recently been shown to be mediated by CaM kinase (13, 14). In whole cell electrophysiologic experiments, Ca^{2+}-mediated activation of lymphocyte and epithelial cell chloride channels was blocked with CaM kinase-specific inhibitory peptides and mimicked by infusion of activated CaM kinase via the patch pipet. In excised patch experiments on single lymphocyte channels, bath perfusion of CaM kinase also activated the chloride channel (13). CF airway epithelial cell lines which were deficient in chloride channel activation by the cAMP pathway were activated by Ca^{2+} ionophore or by injection of activated CaM kinase via the patch pipette (14). Because this parallel pathway to activation of chloride channels bypasses the defective signaling in cystic fibrosis, activation of human epithelial cell CaM kinase by increasing intracellular Ca^{2+} via adenosine receptors is being pursued as a therapeutic approach in this disease (15). We report here the cDNA structure, tissue expression, and analysis of two new isoforms of CaM kinase, the first to be cloned from a nonneuronal or human tissue.

EXPERIMENTAL PROCEDURES

Isolation of Human CaM Kinase cDNAs—Total RNA was isolated from human Jurkat T cells using guanidinium thiocyanate lysis followed by CsCl centrifugation (16, 17). One microgram of total RNA served as template for cDNA synthesis by avian myoblastosis virus reverse transcriptase (Boehringer Mannheim) primed with oligo(dT) (18). One microliter of this reaction was then used as template in 40 cycles of amplification (60 s at 94 °C, 90 s at 46 °C, 120 s at 72 °C) with primers (degenerate positions indicated by parentheses) CK1.S: 5′CCGGTCGACTTTGCGGCCGCTTGG(AGCT)AA(AG)GG3′; COOH.A: 5′GCCGTCGACAAAGTA(AG)AA(CT)(CT)T(AG)TG(AG)AA(AG)TC3′. These primers correspond to the following regions of the rat brain (γ_A) CaM kinase: nucleotides 55–68 (CK1.S) and nucleotides 1324–1345 (COOH.A). The product of this reaction was subcloned via *Sal*I sites on the primers and sequenced. This subcloned PCR product was designated clone C, and its 5′ end did not extend fully to the primer but was truncated due to the presence of an intrinsic *Sal*I site in the human CaM kinase. The insert from clone C was ^{32}P-labeled and used to screen a Jurkat T cell cDNA plasmid library (gift of Naoko Arai, DNAX Research Institute) as described (17). A single cDNA clone of insert size 2.2 kb was isolated, sequenced and designated clone B. Anchored PCR (19) was used to isolate the 5′ end of the human lymphocyte γ CaM kinase. The specific target was amplified under the following conditions: total RNA from Jurkat T cells was reverse transcribed using oligo(dT) as primer and purified on a Centricon-100 microconcentrator (Amicon). Terminal deoxynucleotide transferase (U. S. Biochemical Corp.) was used to tail the cDNA with dATP, and the tailed cDNA was amplified in the presence of two sense primers: 10 pmol oligo(dT)-adapter: 5′AAGGATCCGTCGACATCGATAATACGACTCACTATAAGGGATTTTTTTTTTTTTTTTT3′ plus 25 pmol of outer primer: 5′AAGGATCCGTCGACATC3′ and 25 pmol of a γ CaM kinase-specific antisense primer: 5′TA(CT)TCTCT(GT)GCCAC(AT)ATGTCTTC3′. Amplification conditions were: 1 cycle of 180 s at 94 °C, 120 s at 46 °C, 600 s at 72 °C followed by 30 cycles of 60 s at 94 °C, 60 s at 55 °C, and 120 s at 72 °C. The presence of specific product was identified on a Southern blot probed with a specific oligomer located 5′ of the antisense primer. One microliter of this anchored PCR reaction was subjected to nested PCR using 25 pmol each of inner primer: 5′GACATCGATAATACGAC3′ and a second γ CaM kinase specific primer: 5′CAAGGTCAAACACGAGG3′ in the same amplification protocol as the first round of anchored PCR. This 5′ segment was digested with *Sal*I (which cut γ CaM kinase at nucleotide 195) and with *Cla*I (the oligo(dT)-adapter includes a *Cla*I site) and subcloned into the bluescript vector. Of 12 clones sequenced, 11 of 12 were independent (one appeared twice) and two were full-length, extending beyond the 5′ translational start site. Due to errors introduced during the 60 amplification cycles required for anchored PCR, many of these 11 clones contained a base pair mutation. However, since no two clones contained the same mutation, an unambiguous consensus sequence could be derived and is reported in Fig. 2. Each of the two full-length clones contained a distinct point mutation; however, in one case the mutation was located in a wobble position (a T was substituted for a C at nucleotide 72) and still encoded the same amino acid (Ala^{24}). This anchored PCR subclone, designated 5′ γ CaM kinase, was used to generate a full-length construct as described below.

DNA Constructs and Mutagenesis—A full-length construct of γ_B CaM kinase was assembled in a trimolecular ligation involving three DNA fragments: vector = the phosphatase-treated 2.9-kb *Cla*I-*Not*I fragment of bluescript; 5′ γ CaM kinase = the 230-base pair *Cla*I-*Sal*I fragment of the anchored PCR subclone described above; 3′ γ CaM kinase = the 1.5-kb *Sal*I-*Not*I fragment of clone B. The *Cla*I-*Not*I fragment (containing the full-length γ_B CaM kinase) of this new construct was excised, and *Eco*RI linkers were added. This 1.8-kb *Eco*RI fragment was then inserted into pSRα.BKS (a modified SRα expression vector (20) in which the *Pst*I-*Kpn*I fragment of SRα was replaced with the *Pst*I-*Kpn*I polylinker region from bluescript), forming a complete γ_B CaM kinase-SRα expression construct of 5.3 kb. To generate a mutant of γ_B CaM kinase in which Thr^{287} is replaced with Asp (T287D), site directed mutagenesis was carried out as described elsewhere (17, 21) using single-stranded DNA prepared in M13 and the mutagenic antisense oligomer: 5′GCAAACACTCCAC ATCCTCCTGACGATGC3′. Mutants were screened and verified by DNA sequencing (22).

Analysis of RNA Expression—Human cell lines and human tissues used as sources of RNA were: T cell, Jurkat cell line (obtained from ATCC); B cell, Epstein-Barr virus-transformed B cell line GM03299 (Coriel Institute for Medical Research, Camden, NJ); tracheal epithelium, SV40-transformed fetal human tracheal cell line 56FHTEo$^-$ (14); colonic epithelium, human colon carcinoma cell line T84 (ATCC); keratinocyte, keratinocyte cell line SCC-13 was the gift of Dr. Hung Nguyen, Stanford Department of Pathology; liver, hepatoma-derived cell line HepG2 (23); neuroblastoma: SH-SY5Y cell line (24); muscle, myotubes isolated from a normal 25-year-old man (gift of Mildred Cho, Stanford Department of Pharmacology); heart, left ventricular wall tissue (gift of Dr. Margaret Billingham, Stanford Department of Pathology). RNA was isolated by guanidinium thiocyanate followed by CsCl centrifugation (16, 17). RNA blot techniques were as described (17) with the ^{32}P random-primed insert from clone B as probe and a final wash of 0.5 × SSC/0.1% SDS at 68 °C. RNase protection was carried out as previously described (17, 25). The RNA probe for the variable region was synthesized with T7 RNA polymerase (Stratagene) and corresponded to nucleotides 835–1217 of γ_B CaM kinase. After overnight hybridization of the RNA probe (10^5 cpm) with 10 μg of total RNA, the samples were digested with 40 μg/ml RNase A and 375 units/ml RNase T1 for 30 min at 23 °C. The RNA was extracted and separated on a 6% polyacrylamide denaturing urea gel.

Kinase Expression and Purification—COS-7 cells were transfected with 15 μg of DNA per 10-cm plate via the calcium phosphate method as described elsewhere (26, 27). Mock transfected cells received 15 μg of the SRα parent vector. Jurkat T cells were transfected by electroporation with a Bio-Rad GenePulser, set at 250 V, 960 μFarads, in a 0.4-cm cuvette, with 300 μl of RPMI at room temperature. 70–80 h after transfection, cells were disrupted by sonication with a water cup sonicator (Heat Systems-Ultrasonics) in a lysis buffer containing 50 mM PIPES, pH 7, 1 mM EGTA, 1 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μM benzamidine, and 10% glycerol. Cell extracts were prepared by centrifugation in a microcentrifuge at 12,000 × *g* for 10 min, and the supernatant was harvested and frozen at −80 °C. Small scale purification starting from 40 mg of total cellular protein was carried out in a three-step procedure (DEAE-cellulose anion exchange, phosphocellulose, calmodulin-Sepharose affinity) as previously described (28), yielding CaM kinase that was approximately 90% pure.

Velocity Sedimentation—Total cytosolic protein from COS-7 cells (~100 μg) or Jurkat T cells (~5 mg) transiently transfected with the indicated constructs was layered together with protein molecular weight markers on top of 4.5 ml of preformed 5–20% sucrose gradients

containing 5% glycerol, 50 mM PIPES, pH 7, 150 mM NaCl, 1 mM EDTA in 0.5 × 2-inch centrifuge tubes as described elsewhere (29). The tubes were spun at 36,000 rpm for 14 h at 5 °C. 150-μl fractions were removed from the top and assayed for protein quantity to identify standards and for CaM kinase activity in the presence of calcium/calmodulin. The sedimentation coefficient of CaM kinase was determined relative to molecular weight markers of known $S_{20,w}$. Based solely on this sedimentation value for CaM kinase, a crude estimate of molecular mass was derived using the following equation (29).

$$S_1/S_2 = (M_1/M_2)^{2/3}$$

Kinase Activity and Autophosphorylation—CaM kinase activity was assessed by using a synthetic peptide substrate, KKALRRQETVDAL, or autocamtide-2 (27). Standard assay mixes contained 50 mM PIPES, pH 7, 10 mM $MgCl_2$, 10 μg/ml calmodulin, 10 μM autocamtide-2, 20 μM [γ-^{32}P]ATP (1 Ci/mmol), and either 0.5 mM $CaCl_2$ (for calcium-stimulated activity) or 1 mM EGTA (calcium-unstimulated or autonomous activity). Autocamtide-2 was omitted from background control assays which contained EGTA and no calcium. Control activity of γ_B CaM kinase was <1% of calcium-stimulated activity. Kinase assays in Fig. 6*b* were performed with 1 mM [γ-^{32}P]ATP (0.16 Ci/mmol). Conditions for autophosphorylation in Fig. 7 were essentially as described elsewhere (28). 100 ng of purified γ_B CaM kinase were used for each lane of Fig. 7*a*. In Fig. 7*b*, each assay was performed in triplicate on 20 μg of total protein from γ_B CaM kinase transfected COS cell extracts. Following the assay, reactions were stopped with trichloroacetic acid, assay tubes were spun at 12,000 × *g* for 60 s, and 40 μl of the supernatant were applied to phosphocellulose paper which was washed and measured for Cherenkov radiation (30).

Additional Methods—Calmodulin-binding proteins were visualized with 1 μg/ml biotinylated calmodulin as previously described (31), followed by detection using avidin and biotinylated horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA) and enhanced chemiluminescence (ECL reagents, Amersham Corp.). DNA sequencing was carried out using dideoxynucleotide termination (22) and Sequenase reagents (U. S. Biochemical Corp.) on double-stranded, CsCl-purified DNA that had been denatured with NaOH according to the manufacturer's specifications.

RESULTS

Isolation of Human CaM Kinase Clones—In cloning human lymphocyte CaM kinases, we chose to use PCR based on previously cloned isoforms, since they contain large segments of high homology. Specifically, we designed oligonucleotides to conserved regions of the γ and δ isoforms because they are found in many nonneuronal rat tissues and were therefore likely to be in lymphocytes. PCR products of the predicted size (~1 kb) were amplified from cDNA of intestinal epithelium, B and T lymphocytes. Clone C (Fig. 1) was subcloned from the PCR product of the Jurkat T cell line. Upon sequencing, this clone was identified as a γ-like CaM kinase. Clone C corresponded to approximately 70% of the rat brain γ CaM kinase (γ_A) but lacked the 5′ and 3′ ends. In order to isolate a full length clone of the human γ CaM kinase, clone C was used as a probe to screen a T cell cDNA library. In this screen, clone C hybridized to a different γ-like CaM kinase cDNA (clone B). We shall use the convention of referring to the γ isoform with two "inserts" in the variable region and the first to be cloned as γ_A. The cDNA from which clone B is derived has a single insert and will be referred to as γ_B whereas the cDNA of clone C which has no inserts will be referred to as γ_C. In addition to a 69-base pair in-frame insertion in the clone B variable region relative to clone C (Fig. 2), there were four nucleotide differences between the clones over the 923 bases of overlap; γ_B nucleotide 306 (T) is C in γ_C, nucleotide 333 (A) is T in γ_C, nucleotide 633 (T) is C in γ_C, and nucleotide 688 (G) is A in γ_C. Of these four differences, three are silent with the most C-terminal change (688) producing a difference at the protein level; amino acid 230 is Ala in γ_B and Thr in γ_C. Although it is likely that the high degree of homology of γ_C to the other γ CaM kinases is maintained at its 5′ and 3′ ends, definition of its full sequence awaits the isolation of a full length clone of this isoform.

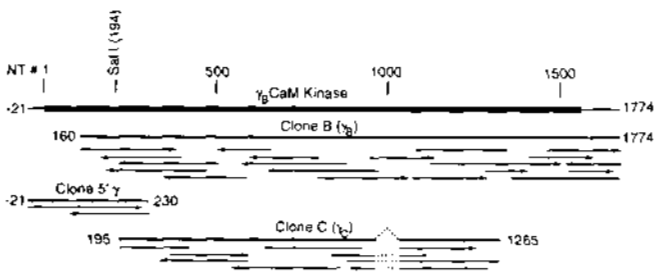

FIG. 1. **Human γ CaM kinase cloning and sequencing strategy.** *Bold line* indicates coding region of γ_B CaM kinase. *Dashed lines* in clone C indicate a 69-base pair deletion relative to clone B. *Arrows* depict direction of DNA sequencing. The *SalI* site was used to join clone B to the 5′ clone and generate a full-length construct.

Because clone B was truncated at the 5′ end by 160 bases and further screening indicated that full length clones were not present in the library, anchored PCR was used to generate additional sequence at the 5′ end. One anchored PCR clone, 5′ γ, was taken to be the 5′ fragment of human γ CaM kinase based on three criteria: (i) the 70 nucleotides which overlap between clone B and clone 5′ γ are identical; (ii) the eleven independent anchored PCR clones sequenced originated from distinct cDNA templates (based on different numbers of As added during the tailing reaction and truncation at distinct 5′ sites) and they predict unambiguous consensus nucleotide and amino acid sequences; and (iii) the catalytic region of CaM kinase to which this clone corresponds is highly conserved and the rat brain γ_A isoform encodes the same amino acid sequence as clone 5′ γ. A full length γ_B CaM kinase construct was assembled using the *SalI* site to link clone B and clone 5′ γ. The predicted molecular mass of γ_B and γ_C CaM kinases based on their cDNA structures are 58,328 and 55,925 Daltons. respectively.

Comparison of Human γ_B and γ_C CaM Kinases to Rat Brain γ_A—Outside of the variable region all three variants of γ CaM kinase are nearly 100% identical in amino acid sequence. Comparison of the highly conserved amino and carboxyl regions of γ with α, β, and δ isoforms yields homologies of ~90% for the amino and ~80% for carboxyl regions (3). γ_B CaM kinase differs from rat brain γ_A by the insertion of a novel 23-amino acid segment in the variable region of γ_B, and the deletion of two segments of 21 and 11 amino acids (Fig. 3). The 23-amino acid insert in γ_B shares 30% identity with the corresponding segment in the β and β' isoforms. The γ_C clone differs from γ_B primarily in that it does not include the 23-amino acid insert.

mRNA Analysis—Although the human γ_B and γ_C CaM kinases were cloned from T cells, RNA blot analysis from the rat suggests γ isoforms may be expressed in multiple tissues (3). Using clone B as a probe for RNA blotting, a 3.9-kb message was detected in 7/7 human cell lines examined (Fig. 4*a*). Because of possible cross-hybridization to highly conserved isoforms of CaM kinase, it is not possible to establish what portion of this signal represents γ_B CaM kinase mRNA. Therefore, RNase protection was carried out using three overlapping probes from the variable region of γ_B CaM kinase, designed to differentiate between expression of γ_B, γ_C, and a putative human γ_A. An ethidium bromide stained gel of the RNA samples used (Fig. 4*b*) shows the RNA isolated from various sources to be of consistent quantity and not degraded. A cartoon of how inserts and deletions in the variable region

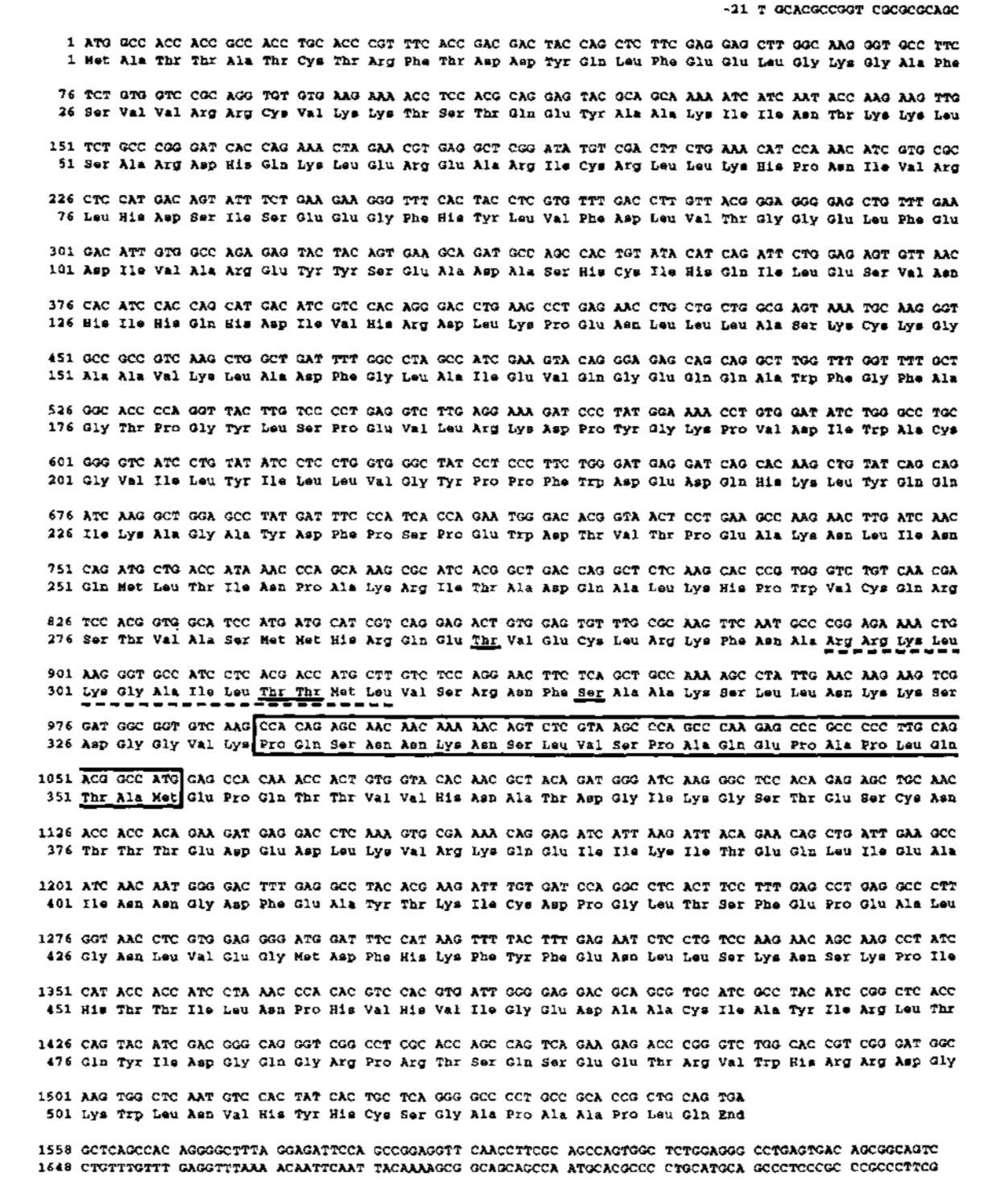

FIG. 2. **Nucleotide and predicted amino acid sequence of γ_B CaM kinase.** Amino acids with *solid underline* are predicted autophosphorylation sites based on similarity to α CaM kinase. The calmodulin binding domain is *underlined in dashes*. The *boxed segment* is present in the γ_B isoform but not in γ_C CaM kinase.

lead to distinct sizes of RNA probe fragments is shown in Fig. 4*c*. Importantly, the nucleotide sequences of γ_B and γ_C are entirely identical over this region except for the 69-base pair insertion in γ_B. Therefore, γ_B would produce a single protected fragment of 382 base pairs, γ_C would produce two protected fragments of 156 and 158 base pairs, whereas γ_A would produce three fragments of 45, 111, and 158 base pairs. For example, protected fragments in Fig. 4*d* from T cell mRNA of 382 and 156/158 base pairs suggest that these cells express both the γ_B and γ_C isoforms. The results shown in Fig. 4*d* are consistent with information obtained from three overlapping variable region RNA probes and one probe from the 5′ end of the gene. In multiple experiments using each of the four probes, transcription of γ_B CaM kinase mRNA was high in T lymphocytes, tracheal and colonic epithelia, keratinocytes, neuroblastoma, and moderately high in muscle. γ_B and γ_C mRNA levels were very low to undetectable in B cells, liver, and heart. The expression pattern of γ_C appeared to mirror

that of γ_B. No evidence reliably emerged suggesting the presence of a putative human counterpart of the rat brain γ_A isoform derived from a common parent gene.

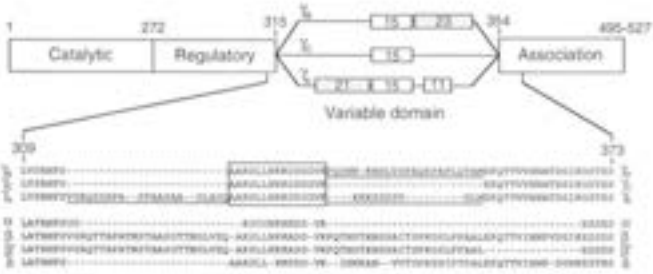

FIG. 3. **Schematic of γ CaM kinase variable domains.** Numbers *inside boxes* indicate size of insertion in amino acids. *Shaded boxes* are unique inserts which correspond to *underlined segments* below. The *unshaded box* is the 15-amino acid segment shared by all three isoforms and is *boxed* below. *Dashes* in amino acid sequence indicate no corresponding residue in that isoform. CaM kinase isoforms α, β, β′, γ_A, and δ are cDNAs derived from rat brain (3, 37, 55, 56).

Expression of γ_B CaM Kinase—Although γ and δ isoforms of CaM kinase have been cloned from rat, they have not been expressed or biochemically characterized. We therefore expressed γ_B CaM kinase and examined whether it had the multimeric structure and autoregulatory activity characteristic of this family of kinases. The level of expression and size of individual subunits of γ_B CaM kinase were examined using biotinylated calmodulin to detect enzyme blotted to nitrocellulose (31). COS-7 cell expression of γ_B CaM kinase yielded a calmodulin binding protein of ~60 kDa and a second protein of ~43 kDa which is likely a proteolytic product (Fig. 5). The smaller product appears to be present *in situ* and not to be produced by proteolysis during cell harvesting based on the fact that transfected cells which were lysed directly in boiling SDS buffer contain the 43-kDa product (data not shown). Recombinant γ_B CaM kinase migrated at the same position as the β isoform of CaM kinase present in purified rat brain CaM kinase (Fig. 5, *Brain*). Recombinant α CaM kinase is included in Fig. 5 for comparison.

Sucrose Density Analysis—CaM kinase from various tissues is multimeric, containing 6–12 subunits per holoenzyme (1).

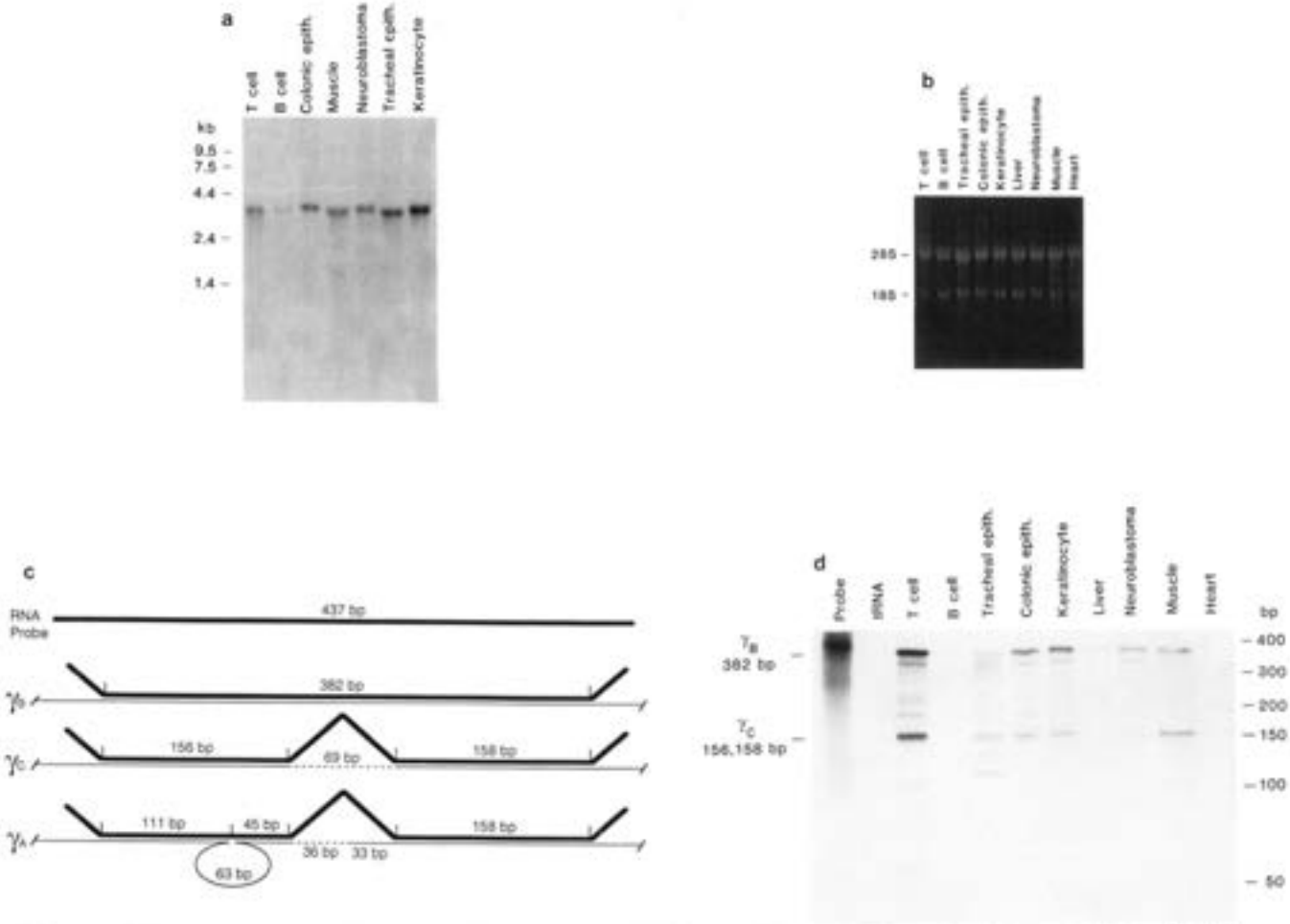

FIG. 4. **mRNA analysis: size and isoform distribution.** *a*, RNA blot. 10 μg of total RNA isolated from the indicated cell lines or tissues were probed with the insert from clone B, indicating a message of about 3.9 kb. *b*, denaturing gel of 10-μg samples of the total RNA used for RNase protection, stained with ethidium bromide. *c*, strategy for RNase protection of variable region of γ CaM kinases. The 382 base pairs of the probe which correspond to γ_B CaM kinase mRNA are from nucleotide 835–1217, or amino acid 279–406. Nonhybridizing segments of the probe are depicted as angling away from the mRNA. *Dashed lines* indicate no sequence corresponding to the probe exists in the mRNA. Predicted sizes of protected fragments are indicated. The presence of a γ_A isoform in the human is hypothetical and could only be detected by this approach if it did not differ in nucleotide structure in shared regions as would be the case in alternative splicing of a common parent gene. *d*, RNase protection. Undigested probe is adjacent to negative control (10 μg of tRNA). In each case 10 μg of total RNA were used. No products consistent in size with predictions for γ_A were reliably observed. This experiment is representative of five separate experiments using four distinct RNA probes.

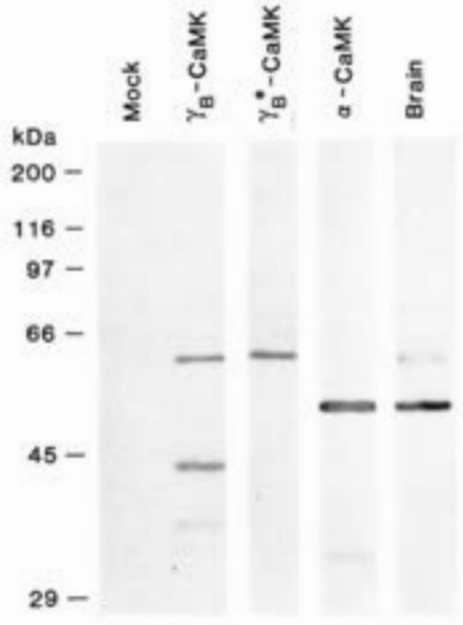

FIG. 5. **Expression of kinase constructs.** *From left*, 25 μg of total cytosolic protein from untransfected COS cells (Mock); COS cells transfected with wild-type γ_B CaM kinase (γ_B-*CaMK*), T287D γ_B CaM kinase (γ_B*-*CaMK*) and wild-type α CaM kinase (α-*CaMK*), or purified rat brain CaM kinase containing both α and β isoforms (*Brain*) was subjected to SDS-polyacrylamide gel electrophoresis, blotted, and detected with biotinylated calmodulin.

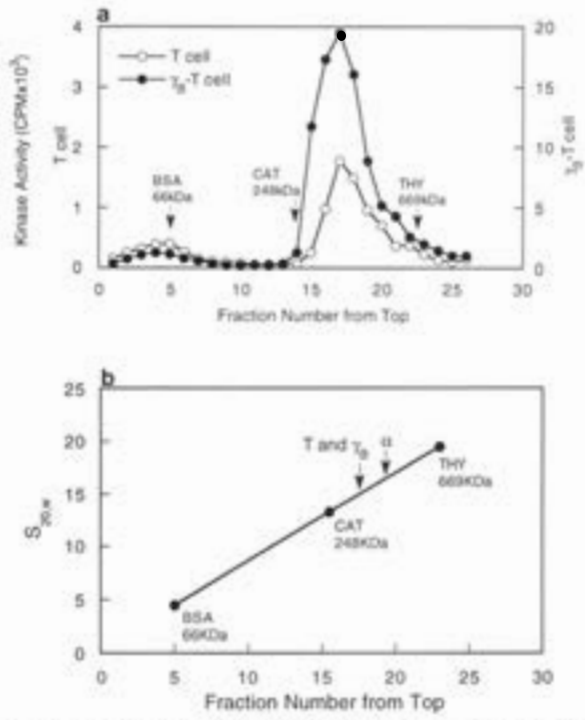

FIG. 6. **Sedimentation velocity analysis.** *a*, sucrose gradients were used to investigate the presence and size of holoenzymes of γ_B CaM kinase transfected in Jurkat T cells as well as endogenous CaM kinase in T cells. Cytosolic extracts (about 5 mg of total protein) were separated by 5–20% sucrose gradients, and fractions were analyzed for CaM kinase activity. Both endogenous and γ_B CaM kinases displayed peak activity in fraction 17. In order to facilitate comparison, endogenous CaM kinase activity is expressed on a scale 5-fold higher than γ_B. Molecular mass marker abbreviations: *BSA*, bovine serum albumin; *CAT*, catalase; *THY*, thyroglobulin. *b*, comparison of sedimentation velocity of holoenzymes: endogenous T cell (*T*), γ_B CaM kinase transfected into either Jurkat or COS-7 cells (γ_B) or α CaM kinase in COS cells (α).

This multimeric structure is believed to be important for its autoregulation (5). We performed sucrose density gradient analysis to assess whether recombinant γ_B CaM kinase forms a holoenzyme and to compare it to the endogenous T cell CaM kinase. Transfection of γ_B cDNA into T cells elevated the CaM kinase activity to 10-fold higher than the endogenous activity present in these cells. The recombinant γ_B enzyme sedimented on sucrose gradients primarily as a single peak of activity with an $S_{20,w}$ = 14.0, whereas our measurement for recombinant α CaM kinase was 16.4 S (Fig. 6, *a* and *b*). These data indicate that, like the neuronal CaM kinases which form holoenzymes of 8–12 subunits (4, 32, 33), γ_B CaM kinase also forms a large multimeric structure. The sedimentation behavior of the recombinant CaM kinase is essentially identical to the endogenous T cell CaM kinase (Fig. 6*a*). Relative to transfection into T cells, the CaM kinase expression level in COS cells was approximately 50-fold higher. This is likely due to the presence of the large T antigen in COS cells, making expression from the SV40-based SRα promoter more efficient (20). A similar analysis of the γ_B CaM kinase expressed in COS cells shows it to sediment at two peaks of equal activity at 4.5 and 14.0 S (data not shown). By SDS gel and calmodulin blot analysis of sucrose gradient fractions, the slower sedimenting peak at 4.5 S is predominantly the 43-kDa fragment sedimenting as a monomer, with some (~30%) full-length γ_B CaM kinase subunits also apparently sedimenting as monomers. The faster peak corresponds to a holoenzyme formed in COS cells with a sedimentation rate identical to the enzyme expressed in T cells (data not shown).

Autoregulation of γB CaM Kinase—Multifunctional CaM kinases which have been characterized to date display two phases of autophosphorylation, a Ca^{2+}-dependent phase which makes the enzyme partially autonomous (by Thr^{286} autophosphorylation) and a subsequent Ca^{2+}-independent phase causing phosphorylation at other sites (1, 9, 34). We purified γ_B CaM kinase from COS cells (see "Experimental Procedures") and examined this kinase for these characteristics. Incubation for 60 s in the absence of calcium/calmodulin caused no perceptible incorporation of ^{32}P, while 20 s in the presence of Ca^{2+}/calmodulin caused moderate incorporation of label. When a 20-s incubation with calcium was followed by 40 s in the absence of calcium (EGTA), total label was significantly increased and a slower migrating species also appeared, consistent with multiply phosphorylated CaM kinase. A full 60-s incubation in the presence of Ca^{2+}/calmodulin does not yield either as much total ^{32}P incorporation or the slower migrating species characteristic of the two phase incubation (Fig. 7*a*). These findings are consistent with an initial requirement of Ca^{2+}/calmodulin dependent autophosphorylation prior to a Ca^{2+}-independent phase of autophosphorylation as described for other isoforms of CaM kinase (7–10). A faster migrating species (~43 kDa) which appears to be autophosphorylated by Ca^{2+}/calmodulin may be the proteolytic fragment of γ_B CaM kinase visible in Fig. 5 as a calmodulin-binding protein.

Does γ_B CaM kinase become Ca^{2+}-independent following Ca^{2+}-stimulated autophosphorylation? To investigate this, γ_B CaM kinase was autophosphorylated by preincubation for 20 seconds with Ca^{2+}/calmodulin and ATP and the effect on its Ca^{2+}-independent activity was monitored. Indeed, Ca^{2+} pretreatment increased Ca^{2+}-independent activity from ~1% to ~40% (Fig. 7*b*). For α CaM kinase, Thr^{286} (corresponding to Thr^{287} of the γ CaM kinases) serves as the critical autophosphorylation site as shown by studies in which generation of autonomous activity correlated with phosphorylation of this site (9, 35) and by site-directed mutation of Thr^{286} to Ala

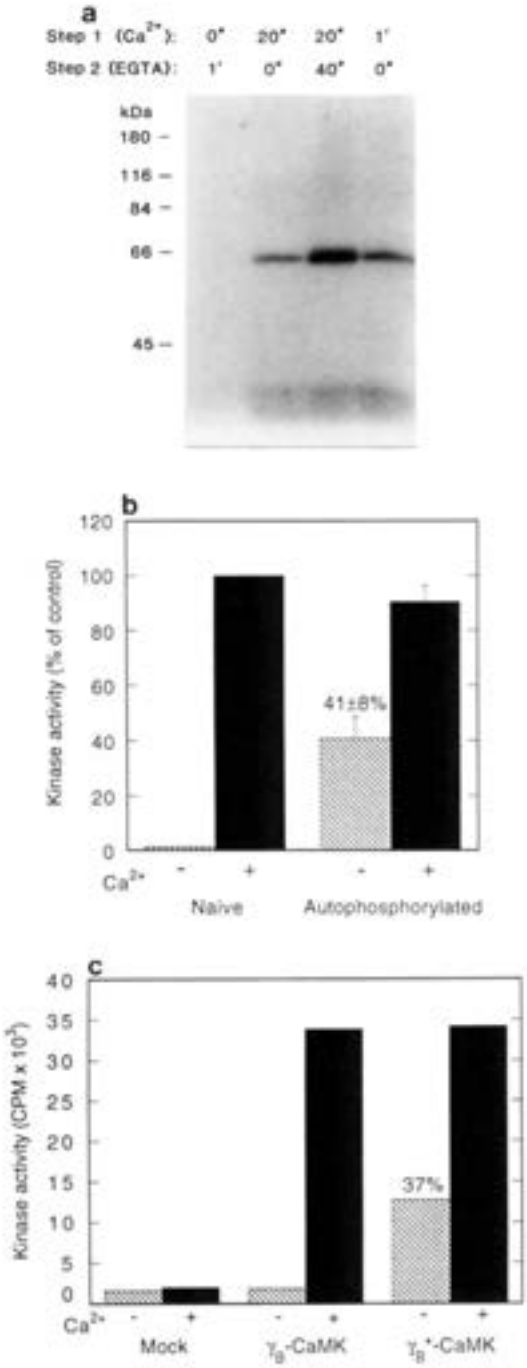

FIG. 7. **Autophosphorylation and activity of human γ_B CaM kinase.** *a*, autoradiograph of autophosphorylated, purified γ_B CaM kinase. Approximately 100 ng of purified γ_B CaM kinase were incubated in the presence of labeled ATP for (*from left to right*): 1 min in the absence of calcium, 20 s in the presence of calcium, 20 s with calcium followed by 40 s without calcium, and 1 min in the presence of calcium. Free calcium was calculated to be below 100 nM for $-Ca^{2+}$, and >1 mM for $+Ca^{2+}$. *b*, generation of autonomy. Cytosolic extract from γ_B-transfected COS cells was incubated in the absence (*Naive*) or presence (*Autophosphorylated*) of Ca^{2+}/calmodulin for 20 s and then assayed for the ability to phosphorylate a CaM kinase-specific peptide. The naive enzyme served as control. These results are the average of three experiments each performed in triplicate. *c*, kinase activity. 4 μg of total protein from cytosolic extracts of transfected COS cells were assayed for phosphorylation of a CaM kinase-specific peptide in the absence or presence of calcium/calmodulin for 30 s, with 1 mM ATP.

which destroyed the ability of the kinase to become autonomous (27). The autoregulatory role of Thr^{286} has also been demonstrated by mutating this residue to aspartic acid, mimicking autophosphorylation at this site, and generating a kinase which is substantially Ca^{2+}-independent (11, 12). We therefore made the analogous mutation in γ_B CaM kinase (referred to as either T287D or γ_B*) to assess whether this threonine has a similar role in the autoinhibitory domain of γ as α. Indeed, the T287D mutation mimics the effect of autophosphorylation in disrupting the autoinhibitory domain. Thus there is a considerable increase in the Ca^{2+}-independent activity from background levels (<5%) for γ_B CaM kinase to nearly 40% of total Ca^{2+}/calmodulin-stimulated activity for the T287D mutant (Fig. 7*c*). Kinase assays of these constructs transfected into COS cells yielded total activity in the presence of Ca^{2+}/calmodulin similar to α CaM kinase (data not shown) and similar "plus calcium" activity for both γ_B and γ_B* CaM kinase (Fig. 7*c*). The concentration of ATP in the assay mix was observed to affect the calcium-independent activity measured, with nearly 40% at 1 mM ATP and 10–15% at 20 μM ATP. This effect is consistent with previous studies (12) and the 1 mM ATP concentration was chosen because it more nearly reflects the intracellular ATP concentration of ~2.8 mM (36). Interestingly, on SDS gels the γ_B* mutant is both perceptibly shifted up and appears to be resistant to proteolysis *in situ* (Fig. 5). The diminished proteolysis of γ_B* and the change in migration may be caused by a conformational change due to the aspartate.

DISCUSSION

CaM kinase has recently been shown to activate a chloride channel in human tracheal epithelia and lymphocytes (13, 14). To understand CaM kinase involvement *in situ* it is important to study the isoforms expressed in these tissues. Here we describe the cDNA structure, tissue localization, and characterization of a CaM kinase expressed in tracheal epithelial cells, lymphocytes, and other human tissues.

We found two closely related variants of CaM kinase transcribed in human, γ_B and γ_C, which differ by 23 amino acids in the variable domain (Fig. 3). It is possible that these two variants arise from alternative splicing as is believed to be the case for β and β' (37, 38). There are, however, four nucleotide differences between γ_B and γ_C which could indicate that these two clones are products of distinct genes rather than of differential splicing of one gene. The fact that three of these four differences do not affect the amino acid encoded suggests that these are not random PCR-generated mistakes (39, 40) although we cannot rule out this possibility. Our results from RNase protection suggest that the γ_B and γ_C isoforms are expressed in most but not all human tissues. In general, expression of γ_C seems to correlate closely with γ_B expression, while we observed no reproducible evidence of a putative human γ_A isoform created by splicing of a parent or closely related γ gene. We do not rule out the possibility that γ_A CaM kinase may exist in humans, as its expression levels may be below our threshold for detection, or it may be present in tissues we did not study. The large difference in transcript levels between B and T lymphocytes (Fig. 4*d*) could reflect isoform-specific regulation relevant to immune function. Although the RNA blot in Fig. 4*a* suggests the presence of substantial quantities of γ_B CaM kinase in B lymphocytes, this signal must actually represent cross-hybridization to related isoforms, since no γ_B or γ_C message is detected by the more specific RNase protection probes (Fig. 4*d*). The RNA blot analysis of rat tissues (3) utilized a γ_A probe which would not have distinguished γ_B and γ_C messages from γ_A. Other

nonneuronal CaM kinases appear to exist in human. In an initial phase of this study we amplified and sequenced three PCR products derived from epithelial cells, T and B lymphocytes which corresponded to the catalytic domain of a CaM kinase closely related to rat brain δ and distinct from the γ-like isoforms described here (data not shown).

Although we have not directly shown that these human γ CaM kinase mRNAs are translated in these tissues, there are at least two links between the γ_B clone and the endogenous CaM kinase activity of T cells. First, both recombinant and endogenous lymphocyte kinases form holoenzymes which cosediment on sucrose gradients. Second, both share characteristics of multifunctional CaM kinases. The endogenous and recombinant T cell kinase activities are dependent on Ca^{2+}/calmodulin and they phosphorylate a synthetic peptide substrate (41) that is phosphorylated by authentic CaM kinase but not by protein kinase C, cAMP kinase, or other cellular kinases.

Recent studies suggest that the holoenzyme structure of CaM kinase may be important in the regulation of kinase activity (5). Although the α and β isoforms of rat brain CaM kinase have been expressed and shown to form holoenzymes, no expression studies have been reported for γ or δ. Sucrose gradient analysis provided evidence that the human recombinant γ_B isoform is capable of forming a holoenzyme composed entirely of homologous subunits which cosediments with the endogenous T lymphocyte CaM kinase. It is interesting that the γ_B isoform expressed in COS cells, but not in its native tissue, is proteolyzed to a considerable degree into a monomeric, catalytically active form. This finding may be a consequence of the >50-fold higher level of expression in COS cells relative to Jurkat T cells, or may be due to an intrinsic stabilization of the holoenzyme in the native tissue.

In order to investigate autoregulation of the γ_B CaM kinase and to create a Ca^{2+}-independent or constitutive mutant, site-directed mutagenesis was employed to replace Thr^{287} with an aspartic acid (T287D or γ_B*). Based on homology to α CaM kinase, the phosphorylation state of this amino acid regulates autonomous kinase activity (1, 11) and the γ_B mutant, γ_B*, was indeed "autonomous," possessing nearly 40% of maximal activity without stimulation by Ca^{2+}/calmodulin (Fig. 7*c*). While Thr^{287} corresponds to the best characterized autophosphorylation site on α CaM kinase (Thr^{286}), several other identified autophosphorylation sites are conserved in γ_B/γ_C CaM kinases, including Thr^{306}, Thr^{307}, and Ser^{315}. These three residues have been shown in the α isoform to become phosphorylated during the second (Ca^{2+} independent) phase of autophosphorylation and to inhibit further binding of Ca^{2+}/calmodulin (28). In analogy to α CaM kinase, it is likely that phosphorylation of these sites following removal of Ca^{2+} is responsible for the higher level of autophosphorylation in *lane 3* relative to *lane 2* of Fig. 7*a*.

Reports that α CaM kinase can regulate transcription via the cAMP response element-binding protein (42), phosphorylate the inositol trisphosphate receptor *in vitro* (43), mediate nuclear envelope breakdown in sea urchin eggs (44), and affect mammalian cell cycle control (45) suggest intriguing ways that CaM kinase can interact with signaling cascades used in many biological systems. Several approaches have been employed to identify the role of CaM kinase in signalling pathways including microinjection of antibody, of highly specific inhibitory peptides or of constitutive forms of the kinase. CaM kinase-mediated activation of chloride channels in human T lymphocytes and airway epithelia was demonstrated using whole cell and single channel patch clamp analysis with inhibitory peptides or purified CaM kinase (13, 14). It is likely that CaM kinase may play a role in some of the many Ca^{2+}-dependent processes for which no mediator has been identified. In the T lymphocyte, for example, Ca^{2+} is involved in multiple processes in addition to chloride channel activation as described above. These processes include activation and lymphokine synthesis (46), negative selection in the thymus (47), clonal anergy (48), and cell death or apoptosis (49). However, identification of the mediator(s) of these calcium signals has been difficult, with success coming only in the case of calcineurin's role in T cell activation (50, 51). Cloning the isoform expressed in a given tissue facilitates further elucidation of CaM kinase function, by approaches such as microinjection or transfection of constitutive mutants (11, 52), and blockage of CaM kinase expression by antisense mRNA or oligonucleotides.

Many questions remain regarding the roles of the multiple isoforms of CaM kinase. In mammalian tissues, with the addition of the two human isoforms reported here, there is a family of seven: α, β, β′, γ_A, γ_B, γ_C, and δ. Where analyzed, these isoforms are found to be regulated developmentally and to exhibit diverse cellular localization (3, 53, 54). Several possible functions can be suggested as to why these variants exist. (i) Isoforms appear to affect holoenzyme assembly. We have shown evidence consistent with fewer subunits being expressed in the γ_B isoform (6–8 subunits) than in α (10–12 subunits). Since autophosphorylation of CaM kinase occurs within each holoenzyme, the number of subunits per holoenzyme may affect the kinetics of autophosphorylation. (ii) Variation between isoforms may affect calmodulin affinity. A recent study suggests that α CaM kinase traps calmodulin by autophosphorylation of Thr^{286}, a mechanism that potentiates its action (6). The modulation of the affinity for calmodulin may be dependent on the nature and position of the insertions since they are adjacent to the C-terminal end of the calmodulin binding domain. (iii) Subcellular localization: the variations between kinase isoforms may contain or alter exposure of protein targeting sequences responsible for intracellular or nuclear localization. This may allow concentration of the kinase near selective cellular substrates. (iv) The intrinsic substrate specificity of the kinase may be modulated, although isoforms from various tissues have been found to possess a highly related *in vitro* substrate profile (1). In summary, although distinct functions have not been characterized for the isoforms yet, their rich variety and differential expression suggest that they have become specialized for roles they play in multiple tissues.

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