Differential functional properties of calmodulin-dependent protein kinase IIγ variants isolated from smooth muscle

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Six variants of calmodulin-dependent protein kinase IIγ were isolated from a ferret-aorta smooth-muscle cDNA library. Variant G-2 is generated by a novel alternative polyadenylation, utilizing a site contained in an intron. The last 77 residues of the association domain are replaced with 99 residues of a unique sequence containing Src homology 3-domain-binding motifs, which alter catalytic activity. Variant C-2 has an eight-residue deletion in an ATP-binding motif and does not autophosphorylate Thr^{286}, but does phosphorylate exogenous substrate. Two variants, B and J, autodephosphorylate. Four variants differing only in the variable domain have differing catalytic activities, despite identical sequences in the catalytic domains. Thus structural features determined by variable and association domains are important for the catalytic activity of calmodulin-dependent protein kinase II.

Key words: alternative polyadenylation, alternative splicing, autodephosphorylation, calmodulin kinase IIγ, oligomerization, smooth muscle.

INTRODUCTION

Calmodulin (CaM) kinases are a family of protein kinases that are activated by Ca^{2+} and CaM. CaM-dependent protein kinase II (CaMKII) is a ubiquitous protein that has a variety of functions in fundamental cellular processes such as metabolism, cell cycle [1], cell shape, gene transcription [2] and in the regulation of ion channel and cytoskeletal function (reviewed in [3–5]). There are four CaMKII isoforms (α, β, γ and δ), each of which is encoded by separate genes. The expression of α and β isoforms is restricted to nervous tissues [6], and their chemistry *in vitro* as well as their function in cells has been extensively studied. Important functional roles of the α and β isoforms in synaptic plasticity, learning and memory have been reported [7]. In contrast, the γ and δ isoforms are expressed in most tissues [8], but very few studies have been carried out to understand their functional roles.

CaMKII consists of an N-terminal catalytic/regulatory domain, a central-linker domain containing variable regions and a C-terminal association domain. The insertion of variable regions within the association domain has also been reported recently [5]. The catalytic/regulatory and association domains show high sequence identity across isoforms. The interaction of association domains between monomers leads to the formation of large multimeric holoenzymes. Based on electron-microscopic reconstructions, the structure of CaMKIIα holoenzyme has been referred to alternatively as a 'hub-and-spoke' pattern [8,9] or a 'gear and foot' pattern [10].

The activity of CaMKII is regulated by autophosphorylation. Upon activation by Ca^{2+}/CaM, Thr^{286} (numbering according to α isoform) is autophosphorylated. This autophosphorylation increases the affinity for Ca^{2+}/CaM and also results in kinase activity in the absence of Ca^{2+}/CaM. Thus autophosphorylation can lead to a prolonged kinase activity in response to a transient Ca^{2+} signal. This property has been suggested to allow CaMKIIα to function as a decoder of Ca^{2+} spike frequencies in cells and also to give the molecule a 'memory'. Two other sites, Thr^{305} and Ser^{314}, can be autophosphorylated in the α and β isoforms after the removal of Ca^{2+}/CaM. Autophosphorylation at Thr^{305} is inhibitory, but at Ser^{314} it has no apparent effect on the affinity for CaM or kinase activity [11].

We have reported previously [12] that a knockdown of CaMKIIγ with an antisense construct led to a significant inhibition of contractility in ferret-aorta smooth-muscle tissue. This study demonstrated a role for CaMKII in the regulation of contractility. The mechanisms involved, probably, multiple variants of CaMKIIγ, which could increase contractility by a number of putative actions, including the activation of extracellular-signal-regulated kinase and the subsequent activation of myosin light-chain kinase [13,14], direct phosphorylation of light chain 20 [15] and effects on channels [16,17].

The aim of the present study was to identify and characterize the CaMKIIγ variants in ferret aorta smooth muscle to gain insights into this ubiquitous, yet little understood, isoform. Thus we present the identification of six different variants of CaMKIIγ formed by alternative splicing and alternative polyadenylation of the same gene. Three of these variants are novel. Autophosphorylation, autodephosphorylation and exogenous substrate phosphorylation were found to differ markedly between variants, including one variant totally lacking in Thr^{286} autophosphorylation. The functional differences have implications for important differences in cellular roles.

Abbreviations used: CaM, calmodulin; CaMKII, CaM-dependent protein kinase II; HRP, horseradish peroxidase; SH3, Src homology 3.

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The nucleotide sequences reported in this paper will appear in GenBank® Nucleotide Sequence Database under the accession numbers AF464182, AF464183, AF464184, AF464185, AF464186 and AF464187 for C-1, C-2, B, J, G-1 and G-2 variants respectively and with accession number AY099467 for the partial genomic sequence of ferret CaMKIIγ.

EXPERIMENTAL

Isolation of cDNAs of CaMKIIγ variants

Reverse transcriptase (RT)–PCR

Total RNA was isolated from 100 mg of aorta by SV Total RNA isolation system (Promega). cDNA was synthesized using 1 μg of total RNA in a 20 μl reaction mixture for 1 h at 42 °C using an RT–PCR kit (ClonTech, Palo Alto, CA, U.S.A.). The reaction product was diluted to 100 μl, and 15 μl of the diluted cDNA was used in a PCR using two degenerate oligonucleotides: (1) sense, 5′-CCTGCACC(C/A)G(C/G)TTCAC(C/A)GA(C/A)GA-3′, and (2) antisense, 5′-T(G/C)A(G/A)(G/A)ATGGC(A/T)CCCTT (C/T/G)AG(T/C)TT-3′, for amino acids CTRFTDD and KLKGAIL respectively in the catalytic/regulatory domain of CaMKII. The PCR amplification was performed in a programmable thermal controller PTC-100 from MJ Research (Waltham, MA, U.S.A.), using a touchdown program as follows: 95 °C/30 s, 60–45 °C/45 s, 72 °C/1 min (16 cycles) followed by 30 cycles of 95 °C/30 s, 52 °C/45 s, 72 °C/1 min. A final extension at 72 °C was applied for 7 min. A product with approx. 900 bp was obtained; this was cloned into TOPO TA vector (Invitrogen, San Diego, CA, U.S.A.) and sequenced.

Screening of cDNA library

Total RNA was isolated from approx. 3 g of aorta using TRIzol® reagent from Gibco BRL (Gaithersburg, MD, U.S.A.). Γ-specific RT–PCR-amplified cDNA was used as the probe in screening a ferret-aorta cDNA library [18]. The procedure followed was essentially identical with the manufacturer's instructions.

Cloning of genomic DNA for CaMKIIγ

Partial genomic DNA for CaMKIIγ was obtained by PCR amplification of ferret liver DNA, isolated by DNeasy tissue kit (Qiagen, Chatsworth, CA, U.S.A.), using two sets of primers: (i) sense, 5′-TAAGATCACAGAACAACTGAT-3′ (P); antisense, 5′-CTGAAACTGCTTCTGGCTAACAGA-3′ (S) and (ii) sense, 5′-AACCTCGTGGAGGGGATGGAT-3′ (Q); antisense, 5′-ACCTCGGTACAGCAGAGACAGACACGA-3′ (R). The positions of the primers are indicated by short arrows in Figure 3.

Northern-blot analysis

RNA was isolated from ferret tissues using TRIzol® reagent (Gibco BRL). Total RNA (30 μg) was loaded on to an 11 cm × 9 cm agarose gel, containing formaldehyde and electrophoresed for 3 h. The gel was blotted to Zeta-Probe membrane (Bio-Rad, Hercules, CA, U.S.A.) by a capillary transfer method using 10 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). The membrane was UV-cross-linked and hybridized at 65 °C with a cDNA probe at high stringency.

Expression and FPLC fractionation of CaMKIIγ variants from COS7 cell lysates

The cDNA coding regions of CaMKIIγ variants were cloned into pcDNA4 His/Max TOPO vector (Invitrogen). Proteins were expressed in COS7 cells by transient transfection using LIPOFECTAMINE™ 2000 (Invitrogen). The expressed cell lysates, in a buffer containing 25 mM Pipes (pH 7.0), 0.2 M NaCl, 1 mM EGTA, 1 mM dithiothreitol and a phosphatase/protease inhibitor cocktail [19], were loaded on to a Superose 12 (Amersham Biosciences) FPLC gel-filtration column and eluted with the same buffer. Fractions (250 μl) were collected and assayed for kinase activity using autocamtide-3 as described below; 3–4 fractions within a peak were pooled for experiments. The molecular masses of the fractions were calibrated using standards (Bio-Rad) containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa), loaded under identical conditions. The positions of the elution of the standards are indicated in Figure 5. In all cases, the 670 kDa fraction contained the CaMKII kinase activity. Western-blot analysis, using a CaMKII antibody, confirmed the presence of CaMKII in the fractions contained in the 670 kDa peak, but not in other fractions or in the 670 kDa fractions of untransfected COS7 cells. For all experiments, the CaMKII variants were normalized to give identical amounts of CaMKII, as identified by Western-blot analysis.

Autophosphorylation

Total autophosphorylation

FPLC-fractionated COS7 cell lysate (4 μg) for the B variant and equivalent amounts of other variants were autophosphorylated in a 50 μl reaction mixture, containing 50 mM Pipes (pH 7.0), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mg/ml BSA, 0.2 mM ATP and 25 μM [γ-^{32}P]ATP. The protein stock contained a phosphatase/protease inhibitor cocktail consisting of 5.5 μM leupeptin, 5.5 μM pepstatin, 20 KIU (kallikrein-inhibitory units) of aprotinin, 20 μM 4-(2-aminoethyl)benzenesulphonyl fluoride, 2 mM Na_3VO_4, 1 mM NaF and 100 μM $ZnCl_2$. To determine the CaM dependence of autophosphorylation, variable amounts of CaM (0–400 nM) were used as indicated in Figure 6(A). For other autophosphorylation experiments, CaM was added to a final concentration of 400 nM. The reaction was incubated at 30 °C for the indicated time and stopped by the addition of SDS/PAGE loading buffer. For time course experiments, aliquots of 50 μl were withdrawn at the indicated time intervals, and the reaction was stopped. Phosphorylated proteins were electrophoresed on 10 % (w/v) polyacrylamide gel and the dried gel was exposed to a phosphor-imaging plate for 24 h. The band intensities were measured by Multianalyst software (Bio-Rad).

Thr^{286} autophosphorylation

Thr^{286} autophosphorylation was performed under the same conditions as described above, except for the use of unlabelled ATP and a CaM concentration of 400 nM. Phosphorylated products were resolved by SDS/PAGE [10 % (w/v) gel]. The gel was transferred on to a PVDF membrane (Millipore, Bedford, MA, U.S.A.) and bands were detected using an horseradish peroxidase (HRP)-coupled secondary antibody and ECL® reagent (Amersham); the image was developed on a phosphor-imaging plate. Intensities of bands were measured using Multianalyst software.

Antibodies

The 310-amino-acid catalytic/regulatory domain of CaMKIIγ was expressed in *Escherichia coli*, and a polyclonal antibody against this peptide was raised in rabbit (Capralogics, Hardwick, MA, U.S.A.). This antibody cross-reacted with all six variant proteins (pan CaMKIIγ) and gave identical results with a commercially available CaMKIIγ antibody (Santa Cruz

Biotechnology, Santa Cruz, CA, U.S.A.) raised in the goat against the C-terminal sequence of CaMKIIγ, with the exception of not recognizing CaMKIIγ_{G-2} that has a unique C-terminal sequence. Phospho-Thr^{286}, the phospho-specific antibody, was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.) and used at a dilution of 1 : 3000.

Substrate phosphorylation

Substrate phosphorylation was measured using an autocamtide-3 peptide [20]. The assay was performed in a 50 μl reaction mixture, containing 50 mM Pipes (pH 7.0), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mg/ml BSA, 5 μg/ml CaM, 20 μM autocamtide-3, 0.2 mM ATP and 25 μM [γ-^{32}P]ATP. For kinase assays of FPLC fractions, 5 μl of each fraction was used after normalization for total protein by a Detergent Compatible protein assay (Bio-Rad) followed by normalization for CaMKII content by Western-blot analysis. FPLC-fractionated CaMKIIγ_B cell lysate (4 μg) and equivalent amounts of the other lysates were used. After incubation at 30 °C for 15 s, the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5 % and processed by spotting on P81 phosphocellulose paper. P81 papers were washed with water and Cerenkov radiation was counted in a liquid-scintillation counter. To determine the time course of substrate phosphorylation, 50 μl of the aliquots were withdrawn at indicated time intervals and spotted directly on P81 phosphocellulose paper and processed.

Two-dimensional gel electrophoresis

Ferret-aorta strips were strung up in an organ bath and tested for viability by contracting with 51 mM KCl physiological saline solution. The strips were then washed with normal physiological saline solution and allowed to relax for 1 h before being quick-frozen in a solid-CO_2/acetone slurry, containing 10 mM dithiothreitol. The strips were homogenized in urea lysis buffer containing 9.5 M urea, 2.0 % (v/v) Nonidet P40, 5 % (v/v) 2-mercaptoethanol, 1.6 % (v/v) Pharmalyte 5–6 and 0.4 % Pharmalyte 3–10. The homogenate (10 μl) was loaded on to a tube gel (as per the Bio-Rad protocol for minigel format two-dimensional gels) followed by 5 μl of overlay buffer (9 M urea/0.8 % Pharmalyte 5–8/0.2 % Pharmalyte 3–10/0.005 % Bromophenol Blue). Protein samples were run on an isoelectric-focusing gel for the first dimension, followed by a 10 % SDS/polyacrylamide gel for the second dimension. Isoelectric-focusing gels contained 9.2 M urea, 4 % (w/v) acrylamide (total monomer), 2 % Nonidet P40, 1.6 % Pharmalyte 5–6, 0.4 % Pharmalyte 3–10, 0.01 % ammonium persulphate, 0.1 % *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. Gels were then transferred on to PVDF membranes and processed by Western-blot analysis. The CaMKII antibody raised against the catalytic domain and against the C-terminal sequences was used at 1 : 1000 and at 1 : 2000 dilution respectively. HRP-labelled secondary antibodies were used at 1 : 2000 dilution. Spots were visualized with ECL® (Supersignal CL-HRP Substrate System; Pierce, Rockford, IL, U.S.A.).

RESULTS

Cloning and characterization of CaMKIIγ subunit variants

CaMKII gene family members show a high degree of homology in their catalytic/regulatory domains. Based on this property, degenerate oligonucleotides were designed from the two ends of these regions. RT–PCR was performed on ferret aortic mRNA. As expected, one single band of approx. 900 bp was amplified. Of the 30 independent clones that were sequenced, 27 were γ and three were δ isoforms. A ferret aortic cDNA library was screened using one of the γ clones as a probe. Fourteen full-length cDNAs were obtained containing six different sequences, which we designated as the C-1, C-2, B, J, G-1 and G-2 variants. The alignment of the translated amino acid sequences is presented in Figure 1. Structures of all six variants are represented diagrammatically in Figure 2. Southern-blot analysis indicated a single copy gene in the genomic DNA (results not shown).

Alternative splicing of the CaMKIIγ gene in the variable region has been described in many different species (reviewed in [5,8]). We have adopted the nomenclature established by others [21–23] in naming the variants that we have isolated. The first reported γ variant, CaMKIIγ_A, was identified to be brain-specific [24]. We did not detect the A variant. Nghiem et al. [22] described a human T-lymphocyte B variant, containing one variable domain. They also described a C variant, containing no variable domain. Singer et al. [21] reported a G variant containing two variable regions, V_1 and V_2, in a porcine cDNA library. They also described the presence of a third variable region V_3. In our six clones, we detected only V_1 and V_2 (Figure 2). Three of our variants represent the ferret homologues of the B, C and G variants described previously, and we have named them as B, C-1 and G-1 respectively. (Simultaneously with the Singer et al. [21] report on the smooth-muscle G variant, a slightly different variant was isolated from tumour cells, but was also named variant G [25].) The remaining three clones are novel variants. One clone contains the V_1 variable region, but not the V_2 region; thus, we have named it as J variant. The remaining two novel clones have variation in the conserved domains, and we have named them as variants C-2 and G-2. Variant G-2 differs from both the G variants described previously.

The C-2 variant is formed by alternative 3′ splice site utilization and lacks part of the ATP-binding region

The C-2 variant has a stretch of eight amino acid residues deleted in the catalytic domain. This deletion overlaps with a part of the consensus motif involved in covering and anchoring the non-transferable phosphates of ATP [26]. The general consensus sequence for this region is (LIV)GxGx(FY)Gx(LIV) (where x represents any amino acid) [26–28]. The corresponding sequence for CaMKIIγ is LGKGAFSVV, which matches the consensus except that Gly at the seventh position has been replaced by Ser [24]. In the C-2 variant, GAFSVV and the subsequent two amino acids Arg-Arg are missing. Inspection of the sequence of the mature mRNA of the other variants indicated the presence of an alternative 3′ site identified by an Ala-Gly, which was utilized as an acceptor in the production of the C-2 variant. As this variant, except for the eight-residue deletion, has the same sequence as variant C-1, we have designated it as CaMKIIγ_{C-2}.

Human CaMKIIγ gene sequence predicts the formation of a C-2 variant by utilization of an alternative 3′ splice site

The human variants B and C are identical (100 % identity in the protein sequence and 95 % identity in the nucleotide sequence) with the ferret variants B and C-1 respectively, prompting us to analyse whether a similar alternative splicing site is present in the human gene. A comparison of the human genomic sequence (Hs10_24193: *Homo sapiens* chromosome 10 working draft sequence segment) with the cDNA sequence of human CaMKIIγ

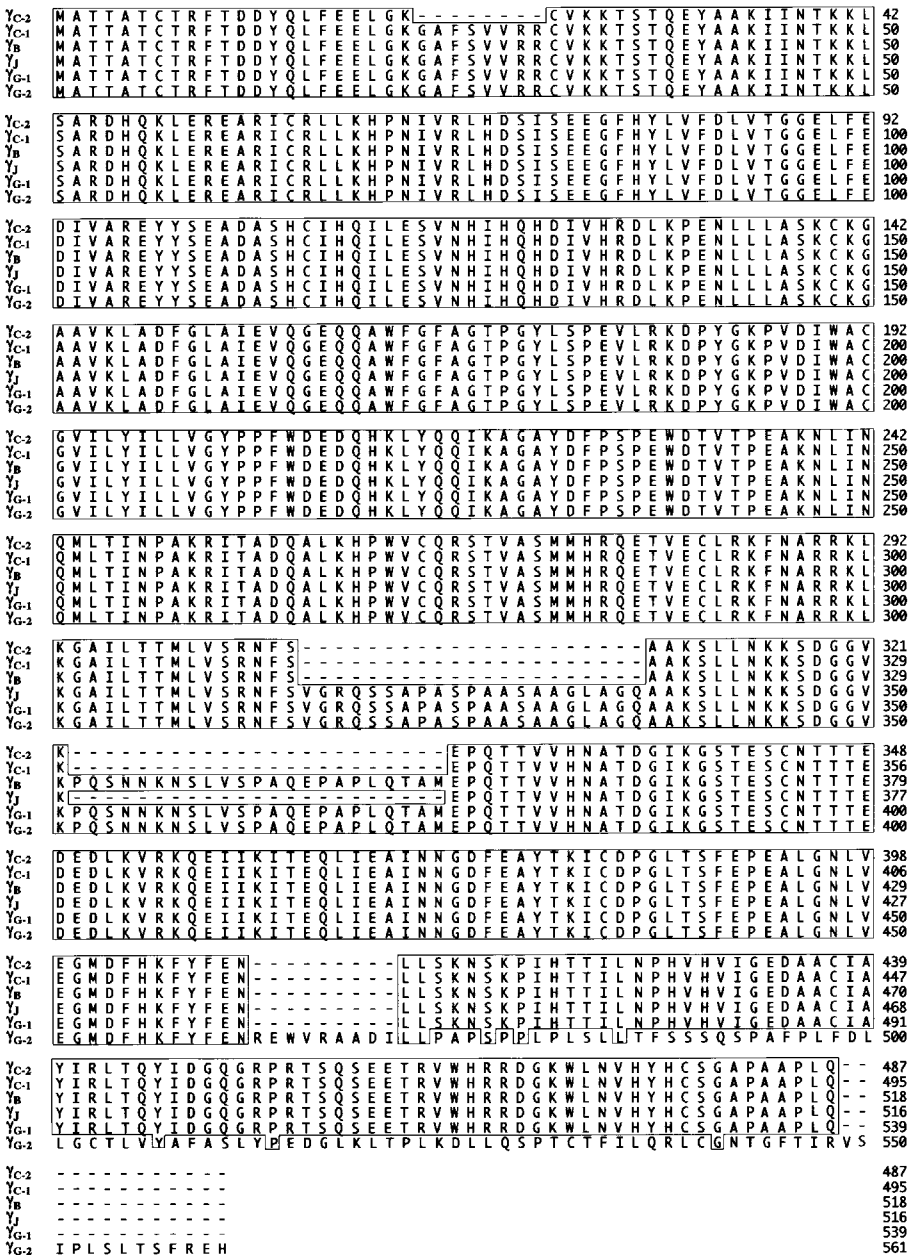

Figure 1 Alignment of translated protein sequences of six variants of CaMKIIγ isolated from a ferret-aorta cDNA library

Identical residues are boxed and broken line represent gaps. The name of the variant is on the left-hand side and the amino acid numbers are on the right-hand side. The GenBank® accession numbers for B, C-1, C-2, J, G-1 and G-2 variants are AF464184, AF464182, AF464183, AF464185, AF464186 and AF464187 respectively.

(accession no. L07044) revealed that there is an intron between the amino acids lysine and glycine in the ATP-binding region as is the case for the ferret gene. In addition, the 24 nt that are deleted in the ferret C-2 variant are identical in the human and ferret genes, including the AG at the splice consensus site. The use of this alternative 3′ splice site would generate a human C-2 variant homologue.

Alternative polyadenylation leads to the formation of a novel association domain in the G-2 variant

The G-2 variant has the N-terminal 457 amino acids in common with variant G-1. This includes 88 amino acid residues in the association domain. However, the C-terminal stretch of 99 amino acids is unrelated to any CaMKII association domain in the databases. We have designated this variant as CaMKII$\gamma_{G\text{-}2}$.

To investigate the origin of the novel sequence in the association domain of CaMKII$\gamma_{G\text{-}2}$, the genomic sequence for that region from the ferret was determined. Oligonucleotides were designed from the novel cDNA sequence of variant G-2 and from the region that was identical across the remaining variants (short arrows in Figure 3A). Inspection of the genomic organization (Figure 3) reveals that there are two introns (331 and 1475 bp) in the association domain in the open reading frame and one (455 bp) in the 3′ untranslated region of the mature mRNA. The extreme downstream antisense primer used was just upstream of the polyadenylated [poly(A)$^+$] tail of the cDNA to obtain the complete sequence. Comparison of the genomic

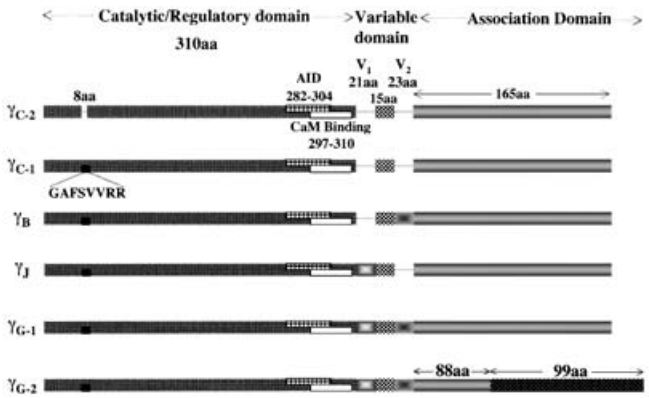

Figure 2 Schematic representation of the protein domain structure of the six CaMKIIγ variants

Broken lines represent gaps. The number of residues in each domain is indicated. The eight amino acid residues missing in the C-2 variant are detailed under the C-1 variant. The novel 99 amino acids in the association domain sequence of CaMKγ_{G-2} are represented by a dotted bar.

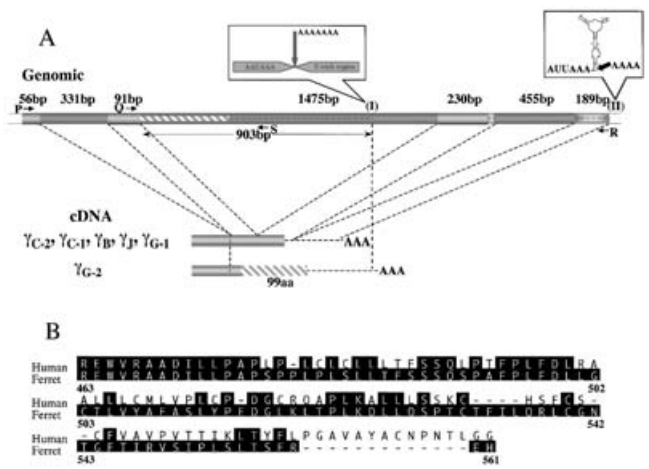

Figure 3 Comparison of genomic and cDNA sequences explains the origin of the γG-2 variant

(**A**) Schematic representation of ferret genomic DNA sequences corresponding to cDNA sequences in the association domain of six CaMKIIγ variants. GenBank® accession number of the partial genomic sequence of ferret CaMKIIγ is AY099467. Top: genomic organization of introns (dark grey) and exons (light grey) showing the number of base pairs in each. Short arrows: primers used to amplify the genomic DNA using PCR. The two alternative polyadenylation sites predicted by the genomic sequence are shown as I and II. The putative regulatory elements for transcription termination and polyadenylation are represented diagrammatically in the box above the genomic sequence. The RNA secondary structure of site II (total free energy = −17.0 kcal/mol; 1 kcal ≡ 4.184 kJ) predicted by a computer program (http://www.genebee.msu.su/) for the 99 bases between AUUAAA and the polyadenylation site II is presented in the box above site II. The arrows with a stretch of A's represent the site of polyadenylation. Bottom: 3′ portion of cDNAs for all variants. Horizontal broken line: the 3′ untranslated region. Exons contributing to the cDNAs are indicated by the broken lines connected to the genomic sequence. The novel sequence in the association domain of the G-2 variant is represented by a grey striped bar. (**B**) Alignment of the novel association domain amino acid sequence of CaMKIIγ_{G-2} of the ferret and the predicted equivalent sequence from the human genome. Alignment was performed by the Clustal method of DNASTAR from LASERGENE. Residues that match the ferret sequence are shaded. Broken line represents gaps. The numbers below the sequences indicate positions in the sequence.

and cDNA sequences revealed that the gene has two alternative polyadenylation sites (I and II) (Figure 3A). All variants except variant G-2 utilize the more of 3′ site II, but variant G-2 utilized the more of 5′ site, located in the intron (site I; Figure 3A). Interestingly, a portion of this intron is retained in the mature mRNA, where it encodes a stretch of 99 amino acid residues (marked by the grey striped bar in variant G-2) in-frame with the preceding exon.

Three *cis*-acting elements in the mRNA sequence work together to determine the 3′-end cleavage and polyadenylation in mammalian pre-mRNA: (i) an AAUAAA (or, less frequently, an AUUAAA) motif which is usually present 10–30 nt but occasionally, up to 300 nt, upstream of the cleavage site; (ii) a less-conserved U- or GU-rich element which is present downstream of the cleavage site, approx. 30 nt downstream of the poly(A)$^+$ site; (iii) the cleavage site where polyadenylation occurs. The details of the transcription termination and polyadenylation mechanism have been reviewed by Zhao et al. [29], Edwalds-Gilbert et al. [30] and Proudfoot [31].

In the case of CaMKIIγ, the *cis* elements for the two alternative polyadenylation sites are quite different. For polyadenylation site I, we found an AAUAAA motif to be present 11 nt upstream of the polyadenylation site and a U-rich sequence 20–30 nt downstream of the polyadenylation site. In contrast, for polyadenylation site II, the AUUAAA motif is present 99 nt upstream of the cleavage site. Long distances between the AAUAAA motif and the cleavage site are accompanied typically by a stem and loop structure, which maintains the spacing required for the binding of the polyadenylation machinery [32]. Consistent with this, the polyadenylation site II sequence is predicted to form a stable stem and loop structure (Figure 3A).

The human CaMKIIγ gene sequence also contains a potential alternative polyadenylation site

The possible formation of a G-2 variant by the use of an alternative polyadenylation site in the human CaMKIIγ gene was also investigated. A 1775 bp intron is present in the human CaMKIIγ gene at the same site as that of the ferret 1475 bp intron (Figure 3). This intron also has an alternative polyadenylation site (AAUAAA), 1129 nt downstream of its 5′ splice site. Interestingly, this intron encodes 104 amino acids in frame with the preceding exon if this alternative poly(A)$^+$ site is used to terminate transcription. The human and ferret amino acid sequences predicted from this region are 72% identical in the first 37 amino acids and less similar in the remaining portion (Figure 3B).

The novel CaMKIIγ_{G-2} has a wide tissue distribution

To determine the tissue distribution of the G-2 variant containing a novel association domain, Northern-blot analysis was performed against total RNA of ferret tissues. For comparison, we first used a non-isoform-specific catalytic/regulatory region as a probe (Figure 4). This probe detected transcripts at the expected position of approx. 2 kb in high abundance in brain, but also in aorta, bladder, stomach and heart. No signal was detected from the liver. On the right panel of Figure 4, the part of the association domain of variant G-2, which comes from the intron, was used as a probe. Signal was detected at the expected position of approx. 2.5 kb in brain, aorta, bladder, stomach and heart. No signal was detected in the liver. The absence of CaMKIIγ variants in the liver has been reported previously [6].

CaMKIIγ_{G-2}, containing a novel association domain, forms oligomers

The association domain of CaMKII is known to be required for oligomer formation [3,33,34]. The unique sequence of the

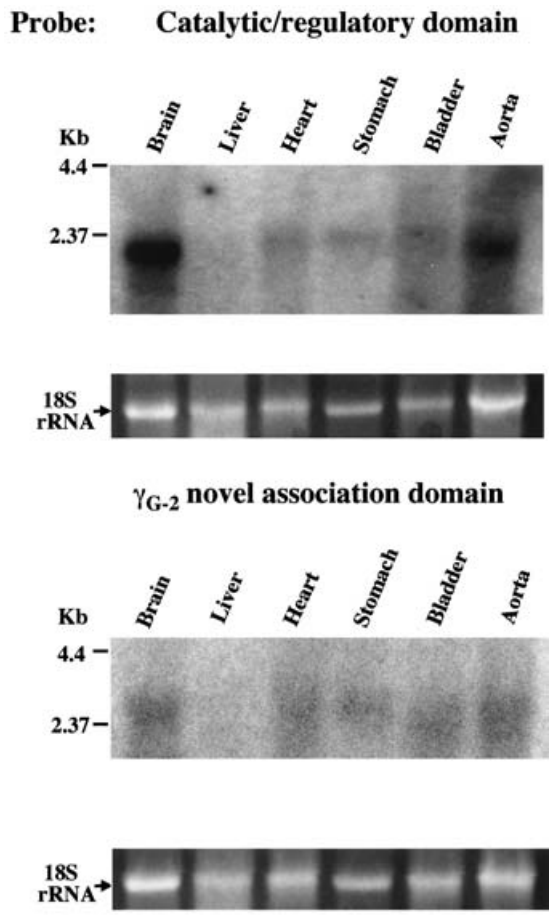

Figure 4 Tissue distribution of CaMKIIγ variants

Autoradiogram of Northern blots using total RNA from six different tissues from the ferret. Upper panel: autoradiogram obtained using a probe directed against the sequence corresponding to the N-terminal 310 amino acids of the catalytic/regulatory region (except C-2 variant). Lower panel: autoradiogram obtained using a probe corresponding to the 99 amino acid novel sequence of CaMKIIγ_{G-2}. Lower panels: ethidium bromide staining of 18S rRNA to compare lane loading of RNA.

association domain of CaMKIIγ_{G-2} raises the question of whether this variant is capable of forming oligomers. The cDNAs of all variants were cloned into a mammalian expression vector (pcDNA4 His/Max TOPO) and expressed in COS7 cells by transient transfection. COS7 cell lysates were loaded on to a Superose-12 FPLC column [21]. The fractions eluted from the column were assayed for CaMK activity. Most of CaMKII kinase activity was present in the fractions corresponding to molecular masses in the range 600–700 kDa, thus indicating that all variants, including variant G-2, form oligomers containing 10–12 monomers (Figure 5). This is consistent with models based on EM reconstruction of CaMKIIα oligomers [10,35].

The question arises as to the possible function of this novel sequence in the association domain of CaMKIIγ_{G-2}. Thus we performed a motif search (http://scansite.mit.edu/) and, at high stringency, the search identified two overlapping Src homology 3 (SH3)-binding motifs within this sequence. A PLCγ-like SH3-binding motif, LLPAPSPPLPLSLLT, centred at Pro^{479} (score, 0.6) and a Grb2-like SH3-binding motif, ILLPAPSPPLPLSLL centred at Pro^{478} (score, 0.5) were found.

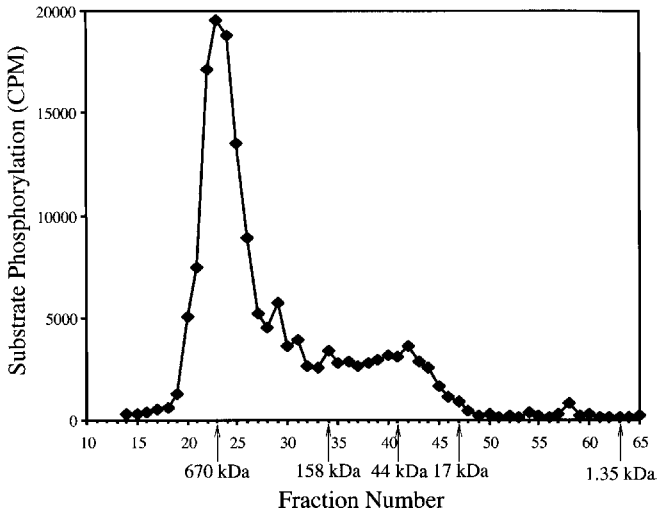

Figure 5 Kinase assay of FPLC fractions predicts the formation of an oligomer of CaMKIIγ_{G-2} in the 670 kDa fraction

Lysates of COS7 cells expressing CaMKIIγ_{G-2} were passed through a Superose 12 FPLC column. The *y*-axis represents the CaMKII activity of each fraction with respect to the specific CaMKII substrate autocamtide-3. The elution of molecular-mass standards under the same conditions is shown by arrows.

Autophosphorylation activity differs amongst CaMKIIγ variants

FPLC-purified COS7 lysates, containing equal amounts of CaMKII variants as judged by Western-blot analysis, were autophosphorylated in the presence of Ca^{2+} and [γ-^{32}P]ATP at varying concentrations of CaM for 15 s, and samples were run on SDS/PAGE gels. Densitometry was performed on the autoradiograms and the band intensities, normalized to maximal intensity, were plotted against CaM concentration (Figure 6A). The different variants have similar CaM sensitivities, i.e. the 50 % maximal [CaM] are between 10 and 50 nM (Figure 6A). Since we found that 400 nM CaM approximates saturation for all variants, we have used this concentration for the subsequent studies.

The above results were normalized as a percentage of the response at 400 nM CaM. In contrast, for absolute values (at 400 nM CaM and 15 s of incubation with [γ-^{32}P]ATP; Figure 6B), it was clear that the amount of autophosphorylation differed dramatically between the variants. Variants B and C-1 showed the highest level of autophosphorylation, variants G-1 and J showed an intermediate amount, and variants C-2 and G-2 showed very low autophosphorylation. A striking difference was seen between variants C-1 and C-2, which differ in sequence only by the deletion of eight residues in the catalytic domain of variant C-2. Also, variants B and G-1 differed considerably in the amount of autophosphorylation at 15 s, even though they differ in domain structure only by the addition of the variable region V1 to variant G-1.

The differences in the amount of ^{32}P incorporated into the variants at saturating [CaM] and at 15 s incubation suggest differences in either the speed or maximal extent of autophosphorylation. Thus autophosphorylation was monitored over a 600 s time course (Figure 6C). Clearly, there are differences among CaMKIIγ variants in the time course and also in the maximal extent of autophosphorylation. Variants B and G-1 approached a steady-state level of autophosphorylation by 600 s of incubation, at which time variant G-1 incorporates about half the amount of ^{32}P compared with that for variant B. This difference is especially of interest since the two variants differ by only one variable domain.

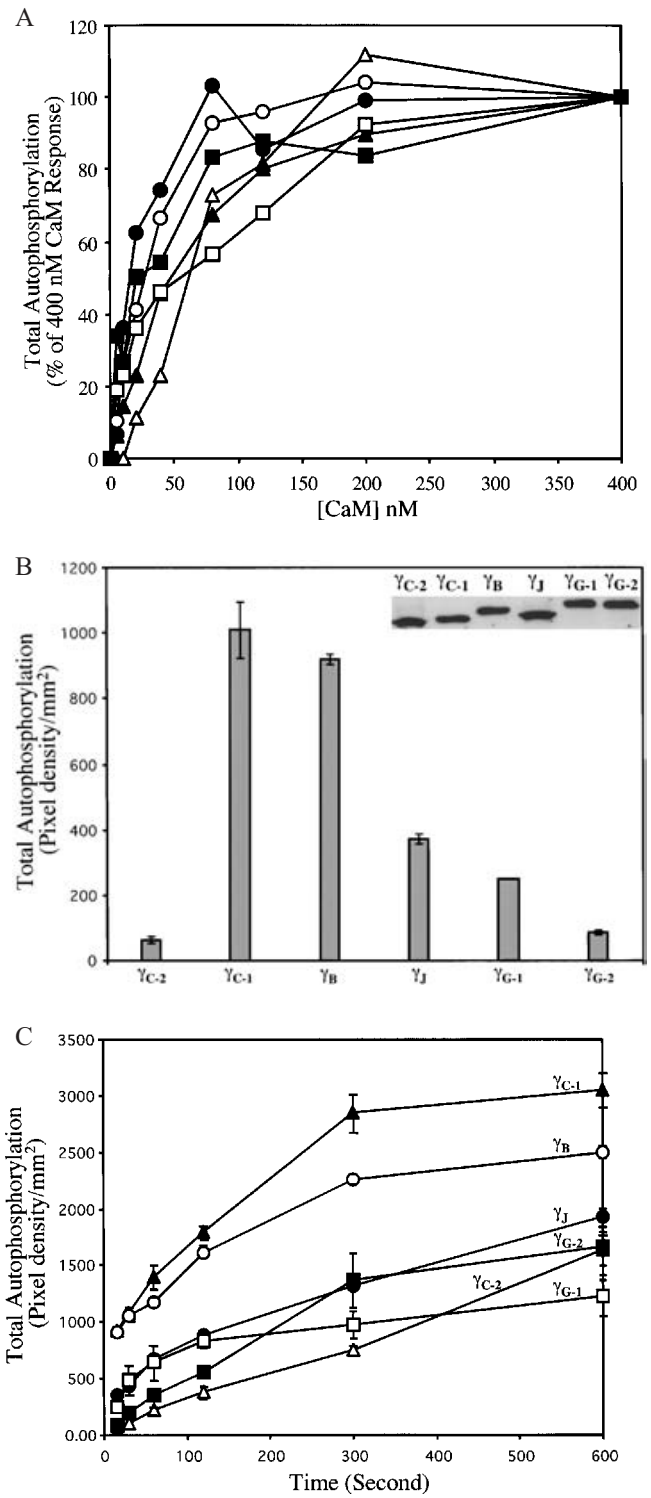

Figure 6 Autophosphorylation at saturating [CaM] is variant-dependent

(**A**) CaM dependence of total autophosphorylation. Autophosphorylation was measured from densitometry of ^{32}P autoradiograms. The extent of autophosphorylation is normalized to that at 400 nM CaM and plotted against [CaM]. Autophosphorylation was measured at 15 s. Equal amounts of CaMKII were used for each assay, as confirmed by Western-blot analysis with a pan-CaMKII antibody (inset, to **B**). △, C-2 variant; ▲, C-1 variant; ○, B variant; ●, J variant; □, G-1 variant; ■, G-2 variant. (**B**) Total autophosphorylation during 15 s incubation at 400 nM CaM. Results are the average of two experiments run at the same time and developed on the same plate, so that the raw numbers are directly comparable. Equal amounts of CaMKII variants were used as confirmed by the Western-blot analysis with a pan-CaMKII antibody (inset). (**C**) Time course of total autophosphorylation of CaMKII variants in the presence of saturating Ca^{2+} and CaM. An average of two experiments performed at the same time and developed on the same plate is shown. The horizontal bars bracketing each data point delimit the range of the two measurements. Equal amounts of CaMKII in each assay were confirmed by Western-blot analysis.

A comparison of variant C-1 versus C-2, which differ only by the eight residues in the catalytic domain, indicates that variant C-2 autophosphorylates more slowly than does C-1.

Autophosphorylation at Thr^{286} depends on the domain structure

Three autophosphorylation sites on CaMKII have been identified in the α and β isoforms: Thr^{286}, Thr^{305} and Ser^{314} (numbering according to the α isoform) [36]. Thr^{286} is the primary site responsible for the autonomous activity of CaM kinases [37]. We have monitored autophosphorylation at Thr^{286} by using a phospho-Thr^{286}-specific antibody. Figure 7(A) shows the Thr^{286} autophosphorylation for CaMKIIγ versus time in the presence of Ca^{2+}/CaM. The average densitometry of two experiments, performed simultaneously so that the values are comparable directly, is shown in Figure 7(B). Note that the Thr^{286} autophosphorylation pattern for several variants is different from that for total ^{32}P incorporation (Figure 6C), indicating that phosphorylation at sites other than Thr^{286} leads to the total ^{32}P incorporation. This is especially of interest, since the assay was performed in the presence of Ca^{2+}/CaM and the likely sites, Thr^{305} and/or Ser^{314}, have been reported to be phosphorylated only after the removal of Ca^{2+}/CaM [36]. Notably, CaMKIIγ_J at 15 s has roughly the same or greater Thr^{286} autophosphorylation as the B variant (Figure 7B) despite the fact that the B variant incorporated roughly twice the total ^{32}P at 15 s (Figure 6C). Thus the B variant must have a higher ^{32}P incorporation into sites other than Thr^{286}. A similar conclusion can be reached with respect to the C-1 variant, which has far more total ^{32}P incorporation than the G-1 variant at 600 s, but less Thr^{286} phosphorylation. Most surprisingly, under these experimental conditions, virtually no Thr^{286} phosphorylation was observed at any given point of time for variant C-2 (Figure 7B), although it shows significant total autophosphorylation (Figure 6C). Thus autophosphorylation of variant C-2 must occur at sites other than Thr^{286}.

Two CaMKIIγ variants containing a single variable domain undergo autodephosphorylation

The time course shown in Figure 7 for all variants indicates that phosphorylation at Thr^{286} for variant B is high at 15 s but then decreases. This occurs despite the presence of high concentrations of phosphatase inhibitors. A similar but less dramatic profile was also seen for the J variant. The common factor in the B and J variants is that each possesses one variable domain. A similar reversal of autophosphorylation has been described previously for the α and β isoforms and has been referred to as autodephosphorylation [38].

Substrate phosphorylation rates are dependent on the variation of domain structure, but do not correlate with autophosphorylation

Autophosphorylation is a measure of kinase activity, but may differ in some aspects from the kinase activity towards an exogenous substrate. The C-2 variant showed no autophosphorylation

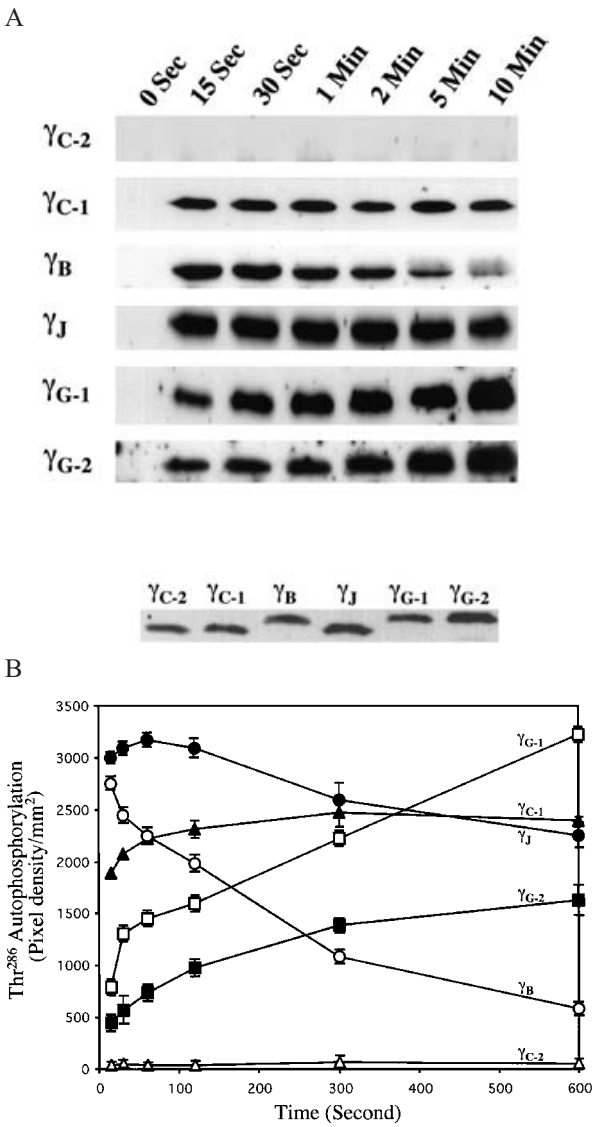

Figure 7 Autophosphorylation at Thr^{286} is variant-dependent

(**A**) Typical immunoblot for Thr^{286} autophosphorylation versus time for CaMKIIγ variants. Phosphorylation detected by a phospho-Thr^{286}-specific antibody. Time points are indicated at the top. Equal amounts of CaMKII were autophosphorylated in the presence of saturating Ca^{2+} and 400 nM CaM. Bottom row: Western-blot analysis of fractionated cell lysates with a pan-CaMKII antibody, confirming equal enzyme loading for all variants. (**B**) Time course of Thr^{286} autophosphorylation. Extent of autophosphorylation as a function of time was measured at saturating Ca^{2+} and 400 nM CaM. Equal amounts of all six CaMKII variants were used and autophosphorylation was detected by phospho-Thr^{286}-specific antibody. Values are the average of two experiments where all time points for each variant were developed on individual plates. The horizontal bars bracketing each data point delimit the range of the two measurements.

at Thr^{286}, but did show a slow phosphorylation at other sites. Thus the question arises whether this variant could phosphorylate an extrinsic substrate. Phosphorylation of a substrate peptide, autocamtide-3 [20], was tested for all variants. This peptide has a sequence taken from the autophosphorylation motif of CaMKII. As shown in Figure 8(A), phosphorylation of autocamtide-3 by variant C-2 is significant (about half of that for variant C-1). The amount of substrate phosphorylation increases monotonically as a function of time for all variants (Figure 8B), even though the time course for autophosphorylation of variants B and J at Thr^{286} decreases with time.

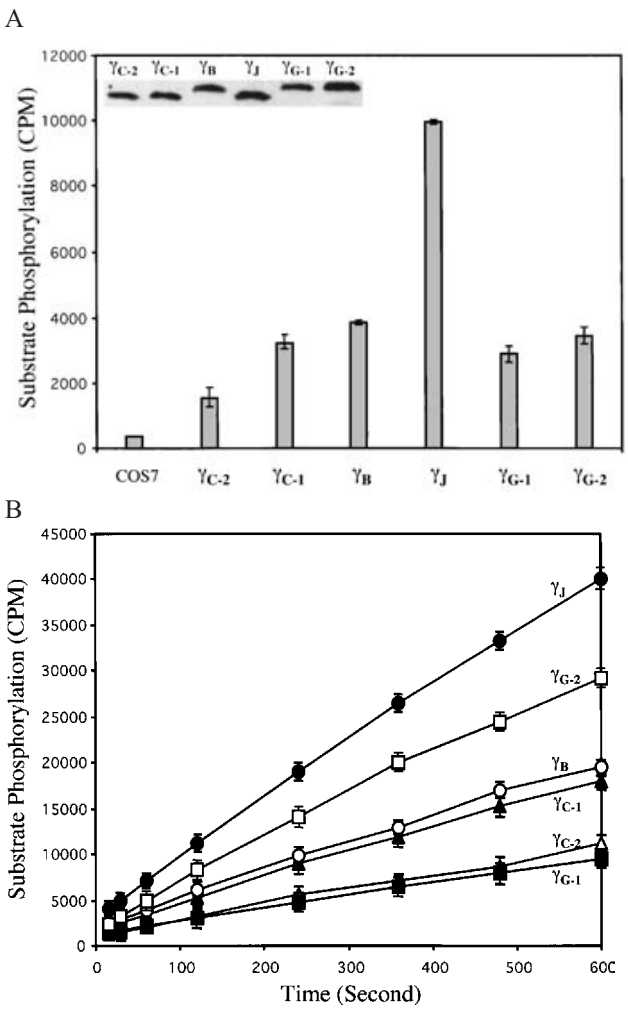

Figure 8 Peptide substrate (autocamtide-3) phosphorylation

(**A**) Relative substrate phosphorylation of autocamtide-3 in comparison with that of a 670 kDa FPLC fraction from uninduced COS7 cell lysate. All results were obtained at 15 s and measured by ^{32}P incorporation into the peptide. Concentrations of all six variants were equal, and uninduced COS7 cell lysate fraction contained equal amount of total protein when compared with that of the γJ variant fraction. Numbers represent averages of two values and the horizontal bars bracketing each data point indicate the range of the values. Variation in uninduced COS7 cell lysate was less than the width of the horizontal bars. (**B**) Time course of substrate phosphorylation. ^{32}P incorporation into autocamtide-3 at time points from 15 to 600 s plotted versus time. Equal amounts of CaMKIIγ fractions were used, as confirmed by Western-blot analysis (inset to **A**). Values are the average of two densitometry experiments performed at the same time and developed on the same plate.

Multiple variants of CaMKIIγ are expressed as protein in ferret-aorta tissue

The molecular masses of the variants range from 55 to 62 kDa, based on the sequence, and furthermore, they run at very similar positions on denaturing SDS/PAGE gels (as can be seen in Figures 6–8). Hence, we used two-dimensional gels to obtain information on the maximum number of possible variants that are expressed as proteins in the tissue from which the cDNA sequences were isolated. Figure 9 illustrates a two-dimensional gel of whole-cell homogenate from ferret aorta, immunostained with an antibody raised against the conserved catalytic domain of CaMKII$\gamma_{C\text{-}1}$ (upper panel) and then re-probed with a second antibody raised against the C-terminal end of conserved C-terminal sequences and specific for γ over δ isoforms (lower panel). The aorta tissue was frozen quickly under resting

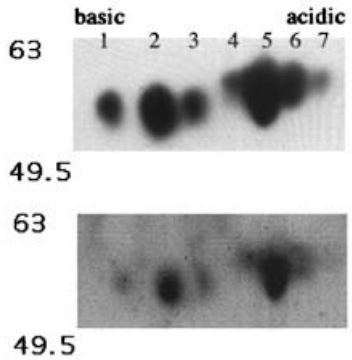

Figure 9 Multiple variants are expressed as proteins in ferret aorta tissue

Two-dimensional gels of whole-cell homogenate of ferret aorta tissue. The tissue was quickly frozen under resting conditions shown previously to minimize autophosphorylation. Immunoblot of the gel was performed with an antibody raised in the rabbit against catalytic domain sequences (upper panel) and then stripped and re-probed with an antibody raised in the goat against C-terminal sequences (lower panel).

conditions, previously shown to minimize autophosphorylation [12]. As can be seen, up to seven variants can be observed. Most of the spots recognized by the antibody raised against the catalytic domain were also recognized by the γ-specific antibody.

DISCUSSION

A major finding of the present study was the discovery of the CaMKII variant G-2, which is formed by an alternative polyadenylation mechanism and contains, as a result, a novel association domain. The DNA sequence for both ferret and human CaMKIIγ contains two alternative polyadenylation sites with specific *cis*-acting sequences for polyadenylation. Apparently, these two polyadenylation sites, of which one is contained in an intron are, however, regulated differentially to produce different variants. Thus CaMKIIγ diversity arises not only due to alternative splicing but also from the alternative polyadenylation.

Regarding the ability of the G-2 variant to oligomerize, it was surprising that, notwithstanding the novel association domain, this protein was still eluted in the same approx. 670 kDa fraction from a gel-filtration column as did the other variants, thus indicating that it can form a multimeric holoenzyme structure. We cannot rule out the possibility that a monomer of variant G-2 formed a complex with other proteins, but it seems unlikely that such a complex would also run exactly in the 670 kDa fraction. On the other hand, interaction of association domains of α and β isoforms has been studied previously by deletion mutation in yeast two-hybrid assay [39], and it was determined that amino acid residues 382–427 and not 427–478 were essential for oligomer formation of the α subunit. The last 51 residues were said to contribute to the stability of the oligomer formation, but were not considered to be essential for oligomer formation [39]. Our results are consistent with the observations of Kolb et al. [39] in that the conserved 88 amino acid residues of the CaMKII$\gamma_{G\text{-}2}$ variant appear to be sufficient for subunit interaction and holoenzyme formation. It is of interest that the transcript for the G-2 variant is present in many different tissues in addition to aorta, from which it was cloned. Further work will be needed to determine the function of this novel 99-amino-acid sequence, but it is of interest that a motif search predicted the presence of two overlapping SH3-binding domains within these 99 amino acid residues. This suggests a possible role for this region in protein–protein interactions regulated by signal transduction pathways in cells.

Another major finding of the present study was the discovery of the C-2 variant that lacks part of the ATP-binding consensus sequence. This deletion in the catalytic/regulatory domain illustrates the fact that alternative splicing of CaMKIIγ can occur in the conserved domains as well as in the variable domain [40]. The study of kinase activity of this variant in comparison with its counterpart, the C-1 variant, showed that both autophosphorylation and phosphorylation of a substrate peptide were impaired significantly in variant C-2. Note that autophosphorylation at Thr^{286} was undetectable in this variant. Given the important role of this autophosphorylation in the 'memory' of the molecule [8], it is predicted that the function of the C-2 variant in cells would differ markedly from the function of CaMKII described previously.

The catalytic domain of all kinases has been described as containing 12 subdomains. Within the N-terminal segment of CaMKII, a consensus motif GxGxxGxV, typical of the majority of known protein kinases, has been described which contributes to the binding of ATP. This specific motif is thought to cover and anchor the non-transferable phosphates of ATP [26]. The deletion in this region in variant C-2 clearly leads to its decreased kinase activity. However, it is of interest that although variant C-2 displayed no detectable autophosphorylation at Thr^{286}, it did incorporate significant ^{32}P into other autophosphorylation sites and did phosphorylate a substrate peptide that mimicked the sequence of the autophosphorylation site, albeit at a slower rate. The residual kinase activity is consistent with the fact that at least four additional segments preserved in variant C-2 are also thought to contribute to ATP binding. An invariant lysine residue of subdomain II has long been recognized as essential for maximal enzyme activity, and is considered to anchor and orient the ATP molecule. This residue is included in the sequence IVHRDLKPENLL, matching a conserved motif typical of most Ser/Thr protein kinases [41], which is preserved in the C-2 variant. The binding of ATP is further stabilized by a glutamic residue of subdomain III. Hydrogen bonding to ATP also comes from residues in subdomains V, VI and VII. Thus in CaMKII$\gamma_{C\text{-}2}$, though a part of subdomain I is deleted, these other interacting residues are preserved and, apparently, are sufficient for the C-2 variant to retain its kinase activity. The inability of the C-2 variant to autophosphorylate Thr^{286} may be related to the fact that the reaction occurs between subunits in the holoenzyme and is therefore under greater steric constraint than is phosphorylation of a peptide substrate [42]. Presumably, this reaction is sterically unfavourable in the C-2 variant.

Our results also suggest that the ATP-recognition consensus sequence in CaMKII has an additional function to facilitate autophosphorylation of Thr^{286}, possibly by contributing in some way to the monomer–monomer interaction. If this region is missing, no Thr^{286} autophosphorylation is seen. This interpretation would be consistent with the results of Lengyel et al. [43], showing that the addition of a synthetic peptide corresponding to this region inhibits autophosphorylation. The peptide in this case may disrupt an endogenous interaction between the subdomain I ATP-binding motif and the Thr^{286} region. Thus taken together, these results suggest that the eight residues of the subdomain I ATP-binding motif and the autophosphorylation site may interact in the three-dimensional structure of the enzyme even though they are distant from each other in the linear sequence.

We also observed in the present study that the insertion and deletion of variable domains into the sequence linking the catalytic/regulatory domain to the association domain leads to alteration in catalytic activity. It is clear that the association domains function in the formation of the holoenzyme structure, and the catalytic and regulatory domains directly control the

phosphotransferase activity of the kinase, whereas the function of the variable domains is not clear. Variable domains might be thought of as simple 'spacers' that determine the length of the 'spoke' in the 'hub-and-spoke' holoenzyme model [8]. However, our study suggests that the organization of variable domains also affects the kinase activity of the catalytic domain. A change in the length of the 'spoke' could easily be imagined to cause an alteration in the interaction of the monomers within the holoenzyme and, hence, alter autophosphorylation activities. However, substrate peptide phosphorylation activities also varied with the organization of the variable domain. This suggests that the variable domain may interact with a part of the catalytic domain in the (as yet undetermined) three-dimensional structure of the CaMKII monomers.

A surprising observation was the 'autodephosphorylation' of variants B and J. A common feature of these two variants is that each possesses a single variable domain. This phenomenon has been reported only once before with respect to the α and β isoforms, and the mechanism was suggested to be a reversal of the kinase reaction [38]. Our study confirms this previous report and extends the observation to the γ isoform. However, the mechanism involving a simple product build-up and a driving of the reaction backwards, as proposed by Kim et al. [38], does not appear sufficient to explain our results. In particular, the decrease in ^{32}P incorporated with time would require not only a reversal of the phosphorylation, which would lead only to a constant steady-state level of autophosphorylation, but also an inhibition of the forward rate after prolonged incubation. Only a combination of the two effects would lead to biphasic time-dependence as observed in Figure 7. It is plausible that autophosphorylation at sites other than Thr^{286} is responsible for this effect.

Because of high sequence homology in the catalytic/regulatory domain in all CaMKII isoforms, it is expected that autophosphorylation will be regulated in a similar manner in all isoforms. As reported for the α and β isoforms, Thr^{286} was autophosphorylated in the presence of Ca^{2+}/CaM in CaMKIIγ, as detected with a phospho-specific antibody. However, our results also indicate that autophosphorylation at other sites of CaMKIIγ also occurs in the presence of saturating levels of Ca^{2+}/CaM. Earlier studies using α and β CaMKII reported that autophosphorylation at Thr^{305} and Ser^{314} occurs only after the removal of Ca^{2+} [36]. The fact that we observe autophosphorylation at sites other than Thr^{286} in the presence of Ca^{2+}/CaM could well be due to differences between the γ isoform and the α and β isoforms.

Thus we have described the *in vitro* function of six CaMKIIγ variants, three of which are novel and one is formed by a novel alternative polyadenylation. These variants differ in their kinase properties in ways that are likely to lead to functional differences in cells. The structure–function differences may also contribute to the yet to be determined three-dimensional structure of CaMKII.

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