Human Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase Kinase \(\beta\) Gene Encodes Multiple Isoforms That Display Distinct Kinase Activity*

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\textsuperscript{‡} The abbreviations used are: CaM, calmodulin; CaMK, Ca\textsuperscript{2+}/CaM-dependent protein kinase; CaMKK, CaMK kinase; ARC, actin-related cytoskeletal protein; CREB, cAMP response element-binding protein; kb, kilobase(s); PCR, polymerase chain reaction; MOPS, 4-morpholinoethanesulfonic acid; GST, glutathione S-transferase; bp, base pair(s); nt, nucleotides; RT, reverse transcription.

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Ca\textsuperscript{2+}/calmodulin-dependent protein kinases (CaMKs) are activated upon binding of Ca\textsuperscript{2+}/calmodulin. To gain maximal activity, CaMK I and CaMK IV can be further phosphorylated by an upstream kinase, CaMK kinase (CaMKK). We previously isolated cDNA clones encoding human CaMKK \(\beta\) isoforms that are heterogeneous in their 3’-sequences (Hsu, L.-S., Tsou, A.-P., Chi, C.-W., Lee, C.-H., and Chen, J.-Y. (1998) J. Biomed. Sci. 5, 141–149). In the present study, we examined the genomic organization and transcription of the human CaMKK \(\beta\) gene. The human CaMKK \(\beta\) locus spans more than 40 kilobase pairs and maps to chromosome 12q24.2. It is organized into 18 exons and 17 introns that are flanked by typical splice donor and acceptor sequences. Two major species of transcripts, namely the \(\beta 1\) (5.6 kilobase pairs) and \(\beta 2\) (2.9 kilobase pairs), are generated through differential usage of polyadenylation sites located in the last and penultimate exons. Additional forms of CaMKK \(\beta\) transcripts were also identified that resulted from alternative splicing of the internal exons 14 and/or 16. These isoforms display differential expression patterns in human tissues and tumor-derived cell lines. They also exhibit a distinct ability to undergo autophosphorylation and to phosphorylate the downstream kinases CaMK I and CaMK IV. The differential expression of CaMKK \(\beta\) isoforms with distinct activity further suggests the complexity of the regulation of the CaMKK/CaMK cascade and an important role for CaMKK in the action of Ca\textsuperscript{2+}--mediated cellular responses.

\(\text{Ca}^{2+}\), an important second messenger in eukaryotic cells, regulates many cellular processes including muscle contraction, neurotransmitter secretion, gene expression, and cell cycle progression (1, 2). Upon stimulation, elevated intracellular \text{Ca}^{2+} mediates its effects via interaction with calmodulin (CaM),\textsuperscript{1} and Ca\textsuperscript{2+}/CaM binds to and induces the activity of a wide range of regulatory proteins. The family of Ca\textsuperscript{2+}/CaM-dependent protein kinases (CaMKs) consists of specific enzymes, e.g. myosin light chain kinase, phosphorylase kinase, and the multifunctional enzymes, such as the various isoforms of CaMK I, CaMK II, and CaMK IV (3–5). The multifunctional CaMKs have been shown to be involved in regulating gene expression by phosphorylating various transcription factors. A number of documents have demonstrated that the CaMK pathway is analogous to the mitogen-activated protein kinase cascade in that it requires an upstream protein kinase, CaMK kinase (CaMKK), to phosphorylate and fully activate CaMK I and CaMK IV (6–11). CaMKK purified from pig brain phosphorylates the threonine residue localized in the “activation loop” of CaMK I (Thr\textsuperscript{177}) and CaMK IV (Thr\textsuperscript{196}), respectively, and increases their activity 20–50 times. Mutation of the Thr residue to Ala abolishes both the phosphorylation and the activation of CaMK I/CaMK IV by CaMKK (12, 13).

Recently, two distinct cDNAs were isolated encoding the rat CaMKK \(\alpha\) and \(\beta\). They share 69\% homology in amino acid sequence and are localized in different regions of the brain (14–19). CaMKK \(\alpha\) is widely distributed in neurons throughout the brain, except in the cerebellar cortex, whereas CaMKK \(\beta\) is relatively restricted in some neuronal populations, particularly in the cerebellar granule cells (17, 18). Like other members of the CaMK family, CaMKK is composed of an N-terminal catalytic domain and a regulatory domain at its C terminus, which contains the CaM-binding site overlapped with the autoinhibitory domain (9, 20, 21). Co-expression of CaMKK with CaMK I or CaMK IV was shown to enhance the activity of CaMK I or CaMK IV toward phosphorylation of cAMP response element-binding protein (CREB) and cAMP response element-dependent reporter gene expression in a Ca\textsuperscript{2+}--dependent manner (15, 19). The CaMKK/CaMK/CREB pathway was recently successfully reconstituted in the \textit{Caenorhabditis elegans} (22). Other than CREB, CaMK IV-mediated signaling is also known to be involved in Ca\textsuperscript{2+}--regulated gene expression through activation of serum response factor (SRF) and activating transcriptional factor-I (ATF-1) (23–25). Intriguingly, CaMKKs and CaMK IV have been shown to exhibit different subcellular localization in the brain. In contrast to the nuclear localization of CaMK IV, both rat CaMKK \(\alpha\) and \(\beta\) are localized in the perikaryal cytoplasm, dendrites, and nerve terminals (18). The distinct subcellular expression patterns suggest the presence of a complicated mechanism for the activation of CaMK IV by CaMKK.
The CaMKK/CaMK IV cascade has also been indicated to interact with the mitogen-activated protein kinase cascade to activate c-Jun NH2-terminal kinase and p38 (26). CaMKK was also suggested to play a role in cell survival. The rat CaMKK α was shown to phosphorylate and activate protein kinase B (PKB) which can then phosphorylate BAD protein. The phosphorylated BAD will then bind to 14-3-3 protein instead of Bclx (PKB) which can then phosphorylate BAD protein. The phosphorylation of these proteins are encoded by the gene CaMKKβ (27).

We previously isolated different cDNA clones corresponding to human CaMKK β that shared more than 90% amino acid sequence homology to rat CaMKK β (28). These cDNA clones are heterogeneous at their 3’-termini. To delineate the transcription of these CaMKK β transcripts, in the present study we examined the genomic structure and transcription of human CaMKK β gene. We found that the human CaMKK β gene contains 18 exons that span more than 40 kb. Multiple transcripts are encoded by the CaMKK β gene through alternative RNA processing. The properties and expression patterns of the various CaMKK β isoforms were investigated.

**EXPERIMENTAL PROCEDURES**

**Human Tissues, Cell Lines, and RNA Preparation**—Human glioblastoma/astrocytoma U-87 MG cells and glioblastoma U-138 MG cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Human non-small lung cancer H-1299 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. These cell lines were maintained in a 5% CO2 humidified chamber at 37 °C. Tissues from normal brains and brain tumors were obtained from patients who underwent surgery at the Veterans General Hospital-Taipei, Taiwan. Informed consent was obtained from each patient. Tissues were snap-frozen immediately after resection. Total RNA was prepared from pulverized tissues or cell lines using the guanidine isothiocyanate method and pelleted through a 5.7 M CsCl cushion (29). The pelleted RNA was dissolved, subjected to DNase I digestion to remove residual DNA, and stored at -80 °C for future use.

5’-Rapid Amplification of cDNA Ends—To extend the 5’ cDNA sequence, a human brain maraton cDNA library was constructed utilizing a Marathon™ cDNA amplification kit according to the manufacturer’s instructions (CLONTECH, Palo Alto, CA). First strand cDNA was synthesized from 10 μg of total RNA prepared from human brain tumor tissue with a CaMKK β-specific primer (5’-CAACTTGACGACACCATAGGAC-3’). The double-stranded cDNA was then amplified by PCR using an adapter primer (5’-CATCCTCATACTGACTCATATAGG-3’) and the gene specific primer under the following conditions: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min for 30 cycles. The PCR product was diluted 10-fold, and 1 μl was used as the template in the nested PCR. The nested PCR primer was performed using a gene-specific nested primer (5’-TCTTGGAGAGACCTTTGGG-3’) and a nested adapter primer (5’-ACTCACTATAGGCTCGAGCCGCCG-3’) under the conditions described above except that the annealing temperature was set at 50 °C. The PCR products were ligated to a pgEM-T vector (Promega, Madison, WI). Colonies containing the CaMKK β cDNA fragment were scored by PCR amplification using CaMKK β-specific sense (5’-TCCTCCCTGGAGGACCATCCACC-3’) and antisense (5’-TCTCTGGAGACCATGGTGCC-3’) primers under the following conditions: 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min for 30 cycles. The positive clones were sequenced in both directions for CaMKK β cDNA sequences using OmniBase™ DNA cycle sequencing system (Promega).

**Northern Blot Analysis**—The CaMKK β and β2-specific cDNA fragments were purified from K5 and K6 clones after restriction digestion with Sac1 (located near the 3’-termini of the CaMKK β cDNAs) and Kpn1 (located within the polycistronic sites of pgEM-T) (28). The Northern terminal common fragment was amplified by PCR using K6 as template with oligonucleotides 5’-AGCTTCTGGAGGTCATC-3’ and 5’-CCAGCCGGCTGACGTAGG-3’ as primers. The cDNA fragments were labeled with [α-32P]dCTP by random priming using the Rediprime DNA Labelling System (Amersham Pharmacia Biotech) and were hybridized to 32P-labeled cDNA probes (10 000 cpm/ml) at 55 °C for 30 min and once with 0.5× SSC and 0.1% SDS for 2 min at 55 °C. Three positive clone, G5-1, G2-1, and G3-1 were isolated upon hybridization to 1× 106 plaques. The genomic DNA fragment was excised by NsiI and subcloned into a pBluescript KS vector for sequencing analysis.

**Purification and Sequencing of the Bacterial Artificial Chromosome (BAC) Clone**—BAC clone 2283L16 was obtained by PCR screening of the BAC library D1 (Research Genetics, Inc.) using CaMKK β-oligonucleotides 5’-CGTATGCTGGACAGAAAGCC-3’ and 5’-TCTTGGACCTCTTCCCTGTC-3’ as primers under the following conditions: 94 °C for 1 min, 50 °C for 30 s and 72 °C for 3 min. Two BAC clones, a single colony of BAC clone was cultured in 5 ml of LB medium containing 12.5 μg/ml chloramphenicol at 37 °C overnight. The overnight culture was transferred into 500 ml of TB medium (12 g bacto-tryptophan, 24 g bacto-yeast extract, and 4 ml glycerol in 1 liter of 0.017 M sodium citrate) and 0.05% SDS at 37 °C for 16–20 h. The BAC DNA was isolated using the alkaline lysis method, treated with RNase A (final concentration 10 μg/ml) for 3 h at 37 °C, and precipitated with 2 μl NaCl and 20% polyethylene glycol 8000. After centrifugation at 13,000 × g for 30 min, the DNA pellet was dissolved in 0.5 mM ammonium acetate, extracted with phenol/chloroform twice, and precipitated by ethanol. After centrifugation, the DNA pellet was washed with 70% ethanol and redissolved in 100 μl of TE (10 mM Tris-Cl, pH 8.0, and 1 mM EDTA) buffer for sequencing analysis.

**Primer Extension Analysis**—The oligonucleotide 5’-GATACGTAC-CACAACCTCTGGCTCCCCGG-3’ was labeled with γ-32PdATP by T4 polynucleotide kinase at 37 °C for 10 min, followed by heat inactivation at 90 °C for 20 min. For primer extension analysis, 2 μg of mRNA prepared from H-1299 or U-87 MG cells was annealed to the labeled primer (0.1 pmol) at 50 °C for 60 min and then reverse-transcribed using the Moloney murine leukemia virus reverse transcriptase (Superscript II, 200 unit, Life Technologies, Inc.) in a 20-μl reaction mixture containing 50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 1 mM dNTP, 20 units RNasin, and actinomycin D (final concentration 50 μg/ml). The reaction was incubated at 42 °C for 50 min followed by heat inactivation at 70 °C for 15 min. The reaction mixture was phenol/ chloroform extracted twice with phenol/chloroform and ethanol precipitated and analyzed on a 6% polyacrylamide sequencing gel together with a sequence ladder obtained by dyeoxy sequencing of the control DNA using the OmniBase™ DNA Cycle Sequencing System (Promega).

**Reverse Transcriptase Analysis of Human CaMKK β mRNAs**—Five μg of total RNA prepared from U-87 MG cells, U-138 MG cells, human placenta tissue, or brain tissues were converted to cDNA by Moloney murine leukemia virus reverse transcriptase (Superscript II, 200 unit, Life Technologies, Inc.) in a 50-μl reaction mixture containing 50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 1 mM dNTP, 20 units RNasin, and actinomycin D (final concentration 50 μg/ml). The reaction was incubated at 42 °C for 50 min followed by heat inactivation at 70 °C for 15 min. The reaction mixture was phenol/ chloroform extracted twice with phenol/chloroform. The reaction product was ethanol-precipitated and analyzed on a 6% polyacrylamide sequencing gel together with a sequence ladder obtained by dyeoxy sequencing of the control DNA using the OmniBase™ DNA Cycle Sequencing System (Promega).
Geneomic Structure and Multiple Transcripts of Human CaMKK β

Two Major Species of Human CaMKK β Transcripts—We previously reported the isolation of two human CaMKK β cDNA fragments, termed K5 and K6, that have different 3′-sequences (28). To further delineate the expression of these two cDNAs, Northern blot analyses were performed using probes derived from the common 5′ region and the 3′-sequences specific to the individual cDNA species (Fig. 1A). A signal of 5.6 kb was readily detected when a human multiple tissue blot was hybridized against the probe derived from the 5′-sequences common to both CaMKK β cDNAs (Fig. 1B). The 5.6-kb mRNA species was highly expressed in brain and to a lesser degree in other tissues. We also noted a weak signal corresponding to 2.9 kb in the brain tissue. With the same blot was differentially hybridized with K5- or K6-specific probes, the 5.6-kb species was detected only by the K5-specific probe; on the contrary, the 2.9-kb species was detected only by the K6-specific probe. Similar results were observed when Northern analysis was performed with RNA prepared from human glioblastoma/astrocytoma U-87 MG cells (Fig. 1C). These results indicated that the two human CaMKK β cDNAs previously identified were derived from two distinct transcripts. Both transcripts are ex-
pressed predominantly in the brain, with the 5.6-kb transcript as the major species. Full-length sequences of these two cDNAs were obtained through a combination of approaches. Rapid amplification of cDNA ends experiments were carried out to further extend the sequences of the 5' end. A BAC clone containing the human CaMKK gene was screened and analyzed for exonic regions encoding the 3' ends. The human expressed sequence tag (EST) database was also searched for entries that match the sequence of human CaMKK. The major transcript (5592 bp), designated as CaMKK1, encodes an open reading frame of 588 amino acids that are identical to the recently published human CaMKK sequence (15). The minor transcript (2960 bp) encodes the CaMKK2, which contains 533 amino acids. The CaMKK1 and 2 share an identical 532 amino acids at the N termini. The kinase catalytic domain and CaM binding domain are located at residues 165–419 and 475–500, respectively. A Pro/Arg-rich region was also identified in the catalytic region at residues 204–225. This region has been suggested to be involved in the recognition of CaMKK with CaMK I/CaMK IV (31). Fig. 2 shows the amino acid alignment of the human, rat, and C. elegans CaMKK isoforms.

The human CaMKK β1 shares 97, 65, and 56% amino acid sequence identity to the rat CaMKK β, rat CaMKK α (19), and C. elegans CaMKK (31), respectively.

Genomic Structure of Human CaMKK —To establish the molecular basis for expression of β1 and β2 transcripts, the genomic structure of CaMKKβ was determined. λ phage clones (G5-1, G2-1, and G3-1) and a bacterial artificial chromosome (BAC number 2283L16) clone that contains sequences corresponding to the human CaMKKβ gene were obtained. Sequence analysis of these genomic clones was performed using oligonucleotide primers derived from the cDNA sequences. The intron-exon boundaries were mapped by sequencing each exon in its entirety along with portions of the adjacent introns (Table I). The approximate size of each intron was estimated by PCR amplification of human genomic DNA using oligonucleotide primers flanking each intron. The human CaMKKβ gene spans more than 40 kb and is organized into 18 exons. A Pro/Arg-rich region was also identified in the catalytic region at residues 204–225. This region has been suggested to be involved in the recognition of CaMKK with CaMK I/CaMK IV (31). Fig. 2 shows the amino acid alignment of the human, rat, and C. elegans CaMKK isoforms.
Genomic Structure and Multiple Transcripts of Human CaMKK β

The exon sequence is represented by uppercase letters, and the intron sequence is represented by lowercase letters. The sequences of exon-intron boundaries were determined by comparing the sequence of genomic DNA and the cDNA sequence of the human CaMKK β gene. The canonical consensus splice sites; ag at the 3' splice site and gt at the 5' splice site, are in boldface. The GenBank™ accession numbers for individual exons are AF321389 (exon 1), AF321390 (exon 2), AF321575 (exon 3), AF321391 (exon 4), AF321392 (exon 5), AF321393 (exon 6), AF321576 (exon 7), AF321577 (exon 8), AF321394 (exon 9), AF321395 (exon 10), AF321396 (exon 11), AF321397 (exon 12), AF321398 (exon 13), AF321399 (exon 14), AF321400 (exon 15), AF321578 (exon 16), AF321401 (exon 17), and AF321402 (exon 18).

**TABLE I**
Exon-intron junction sequences of the human CaMKK β gene

| Splice acceptor sequence | Exon | Exon size | Splice donor sequence | Intron size |
|--------------------------|------|-----------|-----------------------|-------------|
| ctttcctggagGTCGCT         | 1    | 764       | AGCAAGgtaagtgcaca     | ~6.0        |
| tactctctgagGACTGT         | 2    | 530       | AGTCGAGgtagtgagc      | ~3.0        |
| ttttctccagGGCTCC          | 3    | 48        | GGAAGACTtaaatcct      | ~1.2        |
| ttttctccagGCAATG          | 5    | 54        | TACTAATgtagttcct      | ~1.0        |
| tttttctccagGTGCCC         | 6    | 134       | TTTCAAGgtagttgct      | ~3.0        |
| gctgtgagGGAGGCGCCCTG      | 7    | 57        | GGGAGAGGtaggagggg     | ~4.0        |
| ttctcttacagTGTTGG         | 8    | 22        | CCGAGGAgtgcagctc      | ~0.1        |
| gctctcttggagGCCCCG        | 9    | 89        | AGTACTGgagagcagca     | ~1.9        |
| gctctcttggagTACACT        | 10   | 200       | CGGAAAGgaagcctg       | ~0.6        |
| ttttctccagGCTCTG          | 12   | 54        | GGCAGAGtagagggg       | ~3.0        |
| cctctctccagGGCCCA         | 13   | 74        | AGCCAGAGgtagctc       | ~0.3        |
| cctctctccagGCCGGA         | 14   | 88        | ATCAAGAgtacccctg      | ~1.2        |
| tttcttgagGTCGAC           | 15   | 129       | ACCGGAGgtaggag       | ~3.0        |
| cctctcttctagATCCCTG       | 17   | 101       | GCTAGCAGgagggcg       | ~0.6        |
| gggattcctcagCAAAAA        | 16   | 43        | TCTAGCAGtagacccg      | ~0.8        |
| ggtcttggagGAGGCCTG        | 17   | 541 (β2)  | TACAGCAGtaggcctct     | ~3.0        |
| cggattgagGGAAGC           | 18   | 3174 (β1) | AATGAGGGcaagacctg     | ~3.0        |

**FIG. 3.** Genomic structure and transcripts of human CaMKK β, A, the human CaMKK β locus. Exons and introns are drawn schematically and to scale. Exons are indicated by solid boxes and numbered above, whereas introns are indicated by the horizontal line between exons. The dashed lines on top represent the relative size and position of the CaMKK β genomic clones, the BAC clone 2283L16 and the λ phage clones G5-1, G2-1, and G3-1. The restriction endonucleases that cut the BAC clone at the positions indicated are NotI (N) and SauI (S). B, exons encoding CaMKK β (5.6 kb) and β2 (2.9 kb) transcripts. Exons are shown in boxes and numbered above. The hatched boxes represent translated regions, and the empty boxes represent untranslated regions.

Transcriptional Initiation Site(s) of Human CaMKK β—To determine the human CaMKK β transcription initiation site(s), primer extension was performed using mRNA derived from human U-87 MG and H-1299 cell lines as templates and a reverse primer designed from the sequence of the first exon (Fig. 4A). A major extension product of 364 bp was identified with template derived from U-87 MG cells but not with that of H-1299 cells. Several weak extension products were also observed. These results suggest that the transcription start site of human CaMKK β mRNA is 823 bp from the ATG codon and located within the sequences that match the 5'-TGGYY3'-3' (Y, pyrimidine) consensus initiator sequence (Fig. 4B). Sequence analysis of the 5'-flanking region of the human CaMKK β gene revealed that it lacks the canonical TATA box or CAAT box (Fig. 4B). However, the consensus binding sequences for several transcription factors including p300, LyF-1, AML-1, and GATA-1 were identified.

Identification of Alternatively Spliced CaMKK β Transcripts by RT-PCR Analysis—We previously showed that a CaMKK β K6 cDNA variant contained an in-frame deletion of a stretch of 129 nucleotides near its 3'-end (28). Alignment of the cDNA sequence to the genomic sequences revealed that the variant resulted from alternative RNA splicing of exon 14. RT-PCR was
performed to explore the presence of this and additional alternatively spliced CaMKK β transcripts in various human tissues and cell lines (Fig. 5). To detect the transcripts with different 3′-terminal sequences, oligonucleotide F1 located in exon 13 was used as forward primer, and oligonucleotides R1 complementary to the sequences in exon 18 and R2 complementary to the sequences in exon 17 were used as reverse primers. As shown in Fig. 5A, six transcripts (four β1-type and two β2-type) were amplified upon RT-PCR analysis using RNA templates prepared from human placenta (lane 1), human
brain tumor-derived cell lines U-87 MG (lane 2), and U-138 MG (lane 3). These PCR products were individually purified from the gel and subcloned into pGEM-T. Sequence analysis of the β1-related transcripts revealed that one corresponded to the full-length β1 sequence encoded by exons 13–16 plus exon 18, and the others were alternatively spliced variants in which the internal exons 14 or 16 or both were skipped. Similarly, the two β2-related transcripts were identified to be the unspliced β2 transcript encoded by exons 13–17 and an alternatively spliced variant lacking exon 14. Alternative splicing of exon 14 resulted in an in-frame deletion of 43 amino acids, whereas deletion of exon 16 results in a change of the open reading frame that leads to a premature stop of translation. Most of these CaMKK β isoforms were also detected in the brain. Fig. 5B shows the results of RT-PCR analyses of RNA prepared from normal brain tissues (lanes 2 and 3) and brain tumor tissues (lanes 4–7). It was interesting to note that the unspliced full-length β1 transcript represented the predominantly expressed species in the normal brain tissues examined as compared with the alternatively spliced transcripts, whereas the spliced variants appeared to be more abundantly expressed in the brain tumor tissues. In contrast, the full-length β2 transcript and the alternatively spliced β2Δ14 variant were expressed at relatively comparable levels in the normal brain and brain tumor tissues.

Kinase Activity of Human CaMKK β Isoforms—It is well established that CaMK I and CaMK IV are phosphorylated and activated by CaMKK. To determine whether the CaMKK isoforms generated through alternative RNA processing exhibit similar kinase activity to phosphorylate downstream substrates, in vitro kinase assay was performed. The human CaMKK β isoforms were overexpressed in human non-small cell lung cancer H-1299 cells as FLAG-CaMKK fusion proteins. The fusion proteins were immunoprecipitated with monoclonal antibody recognizing the FLAG tag, and the immunoprecipitates were subjected to kinase assay utilizing affinity-purified GST-CaMK I and GST-CaMK IV as substrates. As shown in Fig. 6A, both CaMKK β1 and CaMKK β2 strongly phosphorylate GST-CaMK I in the presence of Ca2+/CaM. The CaMKK β1Δ16 variant retained its kinase activity, whereas phosphorylation of GST-CaMK I by CaMKK β1Δ14 or CaMKK β1Δ14/16 was hardly detectable. Autophosphorylation of CaMK I was observed in the presence of Ca2+/CaM as shown in the mock-transfected sample (lane 2). In parallel experiments, the phosphorylation of GST-CaMK IV by CaMKK β was examined (Fig. 6B). Similar results were obtained, i.e. GST-CaMK IV was phosphorylated by CaMKK β1 or CaMKK β2 in a Ca2+/CaM-dependent manner; deletion of exon 16 did not affect kinase activity, whereas deletion of exon 14 abolished kinase activity. These results showed that CaMKK β isoforms β1, β2, and β1Δ16, although they possess divergent C termini, exhibit similar activity toward phosphorylating the downstream substrates, CaMK I and CaMK IV. In contrast, an in-frame deletion of the internal exon 14 significantly impaired kinase activity.

Autophosphorylation of Human CaMKK β Isoforms—We next examined the autophosphorylation of CaMKK β isoforms using immunoprecipitated FLAG-CaMKK β fusion proteins that were overexpressed in H-1299 cells. As shown in Fig. 7, CaMKK β1 and CaMKK β2 were capable of autophosphorylating themselves. Robust enhancement of autophosphorylation was observed in the presence of Ca2+/CaM. Consistent with the kinase activity of the alternatively spliced CaMKK β variants,
on 9% SDS-polyacrylamide gel and exposed to x-ray film after drying the gel. Plasmids used for transfection included pFLAG-CMV (Mock, p1, p2), and the reaction mixtures were separated by 9% SDS-polyacrylamide gel and exposed to x-ray film after drying the gel.

In the absence of CaM, 2 mM EGTA was added instead of Ca2+/H9262. The CaMKK β isoforms were resuspended in 20 μl of kinase buffer containing 0.1 mM ATP, 5 μCi of [γ-32P]ATP, 4 μg of GST-CaMK I, or 1 μg of GST-CaMK IV in the presence of 2 mM CaCl2 plus 10 μM CaM (lanes 2, 4, 6, 8, 10, 12, and 14). For reactions carried out in the absence of CaM, 2 μM EGTA was added instead of Ca2+/H9262 (lanes 1, 3, 5, 7, 9, 11, and 13). Reactions were incubated at 37°C for 20 min, and the reaction mixtures were separated on 9% SDS-polyacrylamide gel and exposed to x-ray film after drying the gel. Plasmids used for transfection included pFLAG-CMV (Mock, lanes 1 and 2), pFLAG-CMV-CaMKK β2 exon 14 (β2Δ14, lanes 3 and 4), pFLAG-CMV-CaMKK β1 (β2, lanes 5 and 6), pFLAG-CMV-CaMKK β1 (β1, lanes 7 and 8), pFLAG-CMV-CaMKK β1Δ exon 14 (β1Δ14, lanes 9 and 10), pFLAG-CMV-CaMKK β1Δ exon 16 (β1Δ16, lanes 11 and 12), pFLAG-CMV-CaMKK β1Δ exons 14 and 16 (β1Δ14/16, lanes 13 and 14).

Alternative splicing of exon 14 gave rise to CaMKK β proteins incapable of undergoing autophosphorylation, whereas splicing of exon 16 did not affect autophosphorylation activity.

**Chromosomal Localization of Human CaMKK β Locus**—We previously showed that the human CaMKK β gene is located on chromosome 12 by PCR analysis of a human/rodent somatic cell hybrid mapping panel (28). To further map the CaMKK β locus, fluorescence in situ hybridization analysis was performed utilizing biotin-labeled BAC clone as probe. Compared with the 4',6-diamidino-2-phenylindole banding pattern, fluorescent signals of the CaMKK β gene were assigned to chromosome 12q24.2 sub-region.

Fig. 6. Kinase activity of human CaMKK β isoforms. A, phosphorylation of GST-CaMK I. B, phosphorylation of GST-CaMK IV. pFLAG-CMV and the same plasmid expressing individual CaMKK β isoforms were transfected into human non-small cell lung cancer H-1299 cells, respectively. The CaMKK β fusion proteins were shown to be expressed at comparable levels in H-1299 cells. The expressed FLAG-CaMKK β fusion protein was immunoprecipitated with anti-FLAG monoclonal antibody M2 and subjected to kinase assay using bacterially expressed and affinity-purified GST-CaMK I and GST-CaMK IV fusion proteins as substrates. For kinase assay, the immunoprecipitated FLAG-CaMKK β fusion protein was resuspended in 20 μl of kinase buffer containing 0.1 mM ATP, 5 μCi of [γ-32P]ATP, 4 μg of GST-CaMK I, or 1 μg of GST-CaMK IV in the presence of 2 mM CaCl2 plus 10 μM CaM (lanes 2, 4, 6, 8, 10, 12, and 14). For reactions carried out in the absence of CaM, 2 μM EGTA was added instead of Ca2+/H9262 (lanes 1, 3, 5, 7, 9, 11, and 13). Reactions were incubated at 37°C for 20 min, and the reaction mixtures were separated on 9% SDS-polyacrylamide gel and exposed to x-ray film after drying the gel. Plasmids used for transfection included pFLAG-CMV (Mock, lanes 1 and 2), pFLAG-CMV-CaMKK β2Δ14 (β2Δ14, lanes 3 and 4), pFLAG-CMV-CaMKK β2 (β2, lanes 5 and 6), pFLAG-CMV-CaMKK β1 (β1, lanes 7 and 8), pFLAG-CMV-CaMKK β1Δ14 (β1Δ14, lanes 9 and 10), pFLAG-CMV-CaMKK β1Δ16 (β1Δ16, lanes 11 and 12), pFLAG-CMV-CaMKK β1Δ exons 14 and 16 (β1Δ14/16, lanes 13 and 14).

Fig. 7. Autophosphorylation of human CaMKK β isoforms. The CaMKK β isoforms were overexpressed in human H-1299 cells as FLAG-CaMKK β fusion proteins and immunoprecipitated as described in the legend of Fig. 6. For autophosphorylation assay, the immunoprecipitates were resuspended in 20 μl of kinase buffer containing 0.1 mM ATP, 5 μCi of [γ-32P]ATP in the presence of 2 mM CaCl2 plus 10 μM calmodulin (lanes 2, 4, 6, 8, 10, 12, and 14). For reactions carried out in the absence of CaM, 2 mM EGTA was added instead of Ca2+/H9262 (lanes 1, 3, 5, 7, 9, 11, and 13). Reactions were incubated at 37°C for 20 min, and the reaction mixtures were separated by 9% SDS-polyacrylamide gel and exposed to x-ray film after drying the gel.

Fig. 8. Chromosomal localization of human CaMKK β locus by fluorescence in situ hybridization analysis. BAC clone 2283L16, which contains the CaMKK β gene, was biotin-labeled and hybridized to human metaphase slides followed by counterstaining with 4',6-diamidino-2-phenylindole. Two pairs of fluorescent signals were observed, indicating the hybridization of the probe to the two pairs of sister chromatids. The human CaMKK β locus was assigned to the chromosome 12q24.2 sub-region.

alternative splicing of exon 14 gave rise to CaMKK β proteins incapable of undergoing autophosphorylation, whereas splicing of exon 16 did not affect autophosphorylation activity.
The human CaMKK β gene spans a minimum of 40 kb and comprises 18 exons. Multiple transcripts are generated from the human CaMKK β gene through alternative RNA processing. Two major types of transcripts are produced by differential usage of polyadenylation sites located in the last and penultimate exons. Both transcripts contain sequences encoded by exons 1–16 but differ 3’ of this common region. The predominant β1 transcript (5.6 kb in size) skips over exon 17 and splices exon 16 to exon 18 where it polyadenylates. The minor species, β2 (2.9 kb), is produced by inclusion of exon 17, where it concludes its C terminus. Additional forms of transcripts are generated through alternative splicing of the internal exons 14 and/or 16.

Human CaMKK β1 shares 97% amino acid sequence homology to rat CaMKK β. Like rat CaMKK β, the human enzyme is ubiquitously expressed, with the brain as the site showing highest expression (28). In rat brain, CaMKK β displayed an expression pattern distinct from the CaMKK α (15, 18). CaMKK α-immunoreactivity was distributed in neurons throughout the brain, except in the cerebellar cortex. CaMKK β-immunoreactivity was relatively restricted in some neuronal populations. The highest level of CaMKK β was observed in the cerebellar granule cell layer, and moderate immunoreactivity was observed in the cerebral cortex, hippocampal formation, caudate putamen, pontine nuclei, cochlear nucleus, and molecular layer of the cerebellum (15, 18). In the present study, we further examined the regional expression of human CaMKK β in the brain by dot blot analysis using human RNA Master Blot (CLONTECH) to which poly(A)+ RNAs from different regions of the brain were immobilized in separate dots. Our results showed that human CaMKK β was highly expressed in the cerebellum, moderately expressed in the occipital lobe, putamen, subthalamic nucleus, caudate nucleus, frontal lobe, and cerebral cortex, and weakly expressed in the amygdala, hippocampus, medulla oblongata, thalamus, and substantia nigra (data not shown). It appears that the human and rat CaMKK β orthologs encode proteins that are not only structurally similar but also share similar expression patterns. To examine the expression patterns of human CaMKK β and CaMKK β2 transcripts, the human RNA Master Blot was hybridized against β1- or β2-specific probes derived from the unique 3’-terminal sequences of the transcripts. Similar expression patterns were found for both transcripts.

Many genes have been described and characterized that use alternative polyadenylation sites at the 3’-end of their mRNAs according to their cellular environment (32). By a skipped exon mechanism, there are genes that encode two or more mRNAs by using the first alternative 3’-terminal exon with its poly(A) site (pA1) or by skipping that exon entirely and splicing the second 3’-terminal exon into the transcript using pA2 instead (32). By selecting alternative polyadenylation sites, the calcitonin/CGRP (calcitonin-gene-related peptide) gene generates transcripts encoding predominantly calcitonin in thyroid C cells or CGRP in the nervous system (33, 34). Studies of mice with a calcitonin/CGRP transgene showed tissue-specific differences in calcitonin/CGRP expression, suggesting that a specific regulatory mechanism restricted primarily to neurons is required for CGRP expression (35). More recently, the human CUTL1 gene (Cut (Drosophila)-like 1) was shown to give rise to the CDP/Cut (CCAAT displacement protein/human Cut) and CASP (Cut alternatively spliced product) transcripts (36). Both transcripts contain exons 2–14; exon 14 is skipped to exon 15 to generate CDP/Cut transcripts, which contain exons 15–24 or to exon 25 to produce CASP transcript containing exons 25–33 (36). The production of CDP/Cut or CASP mRNA was suggested to depend on the competition between cleavage at the end of exon 24 and splicing between exon 14 and 25 (resulting in the skipping of exons 15–24) (36). It was noted that the polyadenylation signal AAUAAA at the end of exon 24 is embedded within the sequence AAAAUAUUAAA, and the presence of an excess of A residues may lead to inefficient processing of the primary transcripts (37). Therefore, the primary transcripts that are elongated up to exon 33 may invariably be spliced between exons 14 and 25, with the possible cleavage downstream of exon 24 (36). In the present study, the human CaMKK β gene was also processed through the skipped exon mechanism to generate β1 and β2 transcripts with different 3’-termini. The CaMKK β1 encodes 588 amino acids, whereas the CaMKK β2 encodes 533 amino acids. Both contain common exons from 1 to 16 that encode the first 532 amino acids. CaMKK β2 uses exon 17 as its 3’-untranslated region and poly(A) site, whereas CaMKK β1 skips that exon and splices exon 18 into its transcript where it polyadenylates. Consistent with our observation that CaMKK β1 is the predominant transcript, we found that CaMKK β1 utilizes the consensus polyadenylation signal AAUAAA located in exon 18, whereas CaMKK β2 utilizes the atypical polyadenylation signal UAUA located in exon 17. Compared with the AAUAUA motif, the UAUAUA sequence represents a weaker signal for the recognition and binding by CPSF (cleavage and polyadenylation specificity factor) (32). We speculate that the majority of the CaMKK β transcripts are occupied by the CPSF in the AAUAUA site in exon 18 to generate β1 transcripts. The differential processing of primary transcripts from a number of genes through alternative poly(A) site choice has been shown to be a cell cycle-dependent, tissue-specific, or developmentally specific event (32). The regulated expression of these genes may be sensitive not only to the levels of general splicing and polyadenylation factors but also to gene-specific splicing factors that facilitate either the inclusive or the skip-over splice. The detailed mechanism underlying the production of human CaMKK β1 and β2 and the biological significance of this processing event require further study.

Human CaMKK β1 and β2 share identical N-terminal 532 amino acids but differ at their C termini. In the present study, additional forms of CaMKK β transcripts were also identified in human tissues and tumor-derived cell lines that were generated through alternative splicing of the internal exons 14 and/or 16. Skipping of exon 16 leads to a change of the open reading frame yielding a third C terminus that stops prematurely. Skipping of exon 14 leads to an in-frame deletion of 43 amino acid residues (amino acids 442–484) near the C terminus. Alternative splicing is a common mechanism that creates a variety of proteins with constant and variable functional domains from a single gene by RNA processing (38). Members of the CaMK family also contain various isoforms by means of alternative splicing (39, 40). The rat CaMK β is differentially spliced into two isoforms (designated as β1 and β2) with distinct C termini (39, 41). These isoforms are developmentally regulated, with the β1 isoform present in rat embryos from day 18 and the β2 isoform present from day 5 postnatally. More than a dozen alternatively spliced CaMK II transcripts derived from four genes (α, β, γ, and δ) are differentially expressed in different human and rat tissues or cell lines (3, 42–44). By RT-PCR analyses, we demonstrated that CaMKK β1 represented the predominantly expressed species in normal brain tissues, whereas β1A14/16 and β1D16 were more abundantly expressed in brain tumor and placenta tissues. The distinct expression patterns of the unspliced and spliced CaMKK β variants were also observed in two human tumor-derived cell lines, U-87 MG and U-138 MG. In contrast, the relative abun-
dance of β2 and the alternatively spliced β2A14 variant remained unchanged in the tissues and cell lines examined. We also found that CaMKK β1 and β2 exhibit comparable kinase activities to phosphorylate downstream substrate kinases. Deletion of exon 14 did not affect kinase activity, whereas deletion of exon 14 yielded an inactive CaMKK β protein. It is poorly understood how the heterogeneity of the C termini of the CaMKK β isoforms affects its biological function. The C terminus of CaMKK II has been suggested to play a role in its subunit association (20, 21). Whether variant CaMKK β isoforms with different C termini would affect the protein association with itself or other proteins is not clear. Nevertheless, our findings warrant further study to dissect the mechanism that regulates the differential expression of CaMKK β isoforms and to determine the role of CaMKK β-mediated signaling pathways in different tissues under both physiological and pathophysiological conditions.

In the human expressed sequence tag (EST) database, we identified several entries derived from different tissues that contain sequences corresponding to the various CaMKK β transcripts described in this study (the CaMKK β1, Integrated Molecular Analysis of Genomes and their Expression clones 752659 and 824643; CaMKK β2, Integrated Molecular Analysis of Genomes and their Expression clones 2559582 and 2716667; CaMKK β1A14/16, Integrated Molecular Analysis of Genomes and their Expression clone 767832; CaMKK β1A16, Integrated Molecular Analysis of Genomes and their Expression clone 2117038). These findings further support our observations that the human CaMKK β gene is expressed in various isoforms through alternative splicing and polyadenylation. Consistent with our findings, Anderson et al. (15) detect two closely spaced immunoreactive bands in rat brain homogenate by Western blot analysis using antibodies raised against either the N (amino acid residues 28–49) or C (amino acid residues 571–587)-terminal peptides of rat CaMKK β. Similarly, Sakagami et al. (18) also detect two immunoreactive bands at 70 and 73 kDa in rat brain homogenate by Western blot analysis using monoclonal antibody raised against rat CaMKK β peptide (amino acid residues 520–587) (18). In the latter report, the authors further noted that the closely spaced doublets migrated slightly faster than the full-length CaMKK β overexpressed in COS cells. It would be interesting to verify whether the two closely spaced doublets identified in rat brain homogenate represent alternatively spliced variants corresponding to the human CaMKK β isoforms from which exons 14 and/or 16 are removed. The result obtained from this study will also provide us information regarding whether the orthologous human and rat CaMKK β genes are conserved in genomic organization and are expressed through similar post-transcriptional RNA processing event.

Deletion of exon 14 rendered CaMKK β largely inactive upon phosphorylating its downstream targets, CaMK I and CaMK IV. This is likely a result of the interference of its interaction with calmodulin. In a previous study using site-directed mutagenesis and a synthetic peptide, Tokumitsu et al. (45) identify the region of the calmodulin binding site (residues 438–463) in rat CaMKK α. By NMR spectroscopic study, Osawa et al. (46) determine the structure of calcium-bound calmodulin (Caα2/CaM) complexed with the 26-residue peptide corresponding to the CaMKK α CaM-binding site. In this complex, the CaMKK α peptide was found to form a fold comprising an α helix (residue 444–454) and a hairpin-like loop (residue 455–459) whose C terminus folds back onto the helix. Both the α helix and the hairpin-like loop are involved in the interaction of CaMKK with CaM, and Trp 444 and Phe 459 were identified as the anchoring residues to the C- and N-terminal domain of CaM. Mutation of Phe 459 to Asp completely abolished Ca a2/CaM binding. By sequence alignment, the amino acids 475–500 of human CaMKK β correspond to the Caα2/CaM binding site, with Leu 481 and Phe 496 as the anchoring residues, respectively. Alternative splicing of exon 14 gives rise to a CaMKK β variant with an in-frame deletion of residues 442–484, which lacks the first 10 residues (including the C-terminal anchoring residue) of the Caα2/CaM binding site. This may impair Caα2/CaM binding and lead to inactivation of CaMKK β. In Drosophila, up to 18 different alternatively spliced CaMKK II variants with heterogeneous C termini covering the CaM binding domain were generated from a single gene (47). Seven variants were shown to have different binding affinity for CaM (47). These findings support that CaMKK isoforms are expressed in various isoforms through alternative RNA splicing to generate isoforms that exhibit distinct kinase activity and calmodulin binding activity.

In summary, we have demonstrated that the human CaMKK β gene is organized into 18 exons and 17 introns and is localized to chromosome 12q24.2. Multiple transcripts are produced through alternative splicing and polyadenylation. These CaMKK β isoforms, except the ones in which exon 14 is deleted, undergo autophosphorylation in the presence and absence of Caα2/CaM, whereas the binding of Caα2/CaM is required for efficient phosphorylation of the downstream target kinases GST-CaM-I and GST-CaM-IV. The CaMKK β isoforms are differentially expressed in human tissues and cell lines. The diversity of human CaMKK β isoforms with heterogeneous C termini with distinct kinase activity and their relative abundance in different tissues further demonstrate the complexity of the regulation of the CaMKK-CaM signaling pathway and the important role of CaMKK β in Caα2-mediated cellular processes.

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REFERENCES
1. Santella, L. (1998) Biochem. Biophys. Res. Commun. 244, 317–324
2. Berridge, M. J. (1989) Neuron 13, 13–26
3. Hanson, P. I., and Schulman, H. (1992) Annu. Rev. Biochem. 61, 559–601
4. Heist, E. K., and Schulman, H. (1998) Cell Calcium 25, 103–114
5. Means, A. R. (2000) Mol. Endocrinol. 14, 4–13
6. Lee, J. C., and Edelman, A. M. (1994) J. Biol. Chem. 269, 2158–2164
7. Okuno, S., Kitani, T., and Fujisawa, H. (1994) J. Biochem. 116, 923–930
8. Park, I. K., and Soderling, T. R. (1999) J. Biol. Chem. 274, 30464–30469
9. Soderling, T. R. (1999) Trends Biochem. Sci. 24, 232–236
10. Sugita, R., Mochizuki, H., Ito, T., Yokokura, K., Kobayashi, R., and Hidaka, H. (1984) Biochem. Biophys. Res. Commun. 133, 684–701
11. Uemura, A., Naito, Y., and Mumm, D. D. (2000) Biochem. Biophys. Res. Commun. 272, 355–360
12. Selbert, M. A., Anderson, K. A., Huang, Q.-H., Goldstein, E. G., Means, A. R., and Edelman, A. M. (1995) J. Biol. Chem. 270, 17616–17621
13. Haribabu, B., Hoek, S. S., Selbert M. A., Goldstein, E. G., Tomhave, E. D., Edelman, A. M., Snyderman, R., and Means, A. R. (1995) EMBO J. 14, 3679–3686
14. Edelman, A. M., Mitchelli, C. I., Selbert, M. A., Anderson, K. A., Huang, S.-K., Stapleton, D., Goldstein, E. G., Means, A. R., and Kemp, B. E. (1996) J. Biol. Chem. 271, 10806–10810
15. Anderson, K. A., Means, A. R., Huang, Q.-H., Kemp, B. E., Goldstein, E. G., Selbert, M. A., Edelman, A. M., Fremeau, R. T., and Means, A. R. (1998) J. Biol. Chem. 273, 31880–31889
16. Kitani, T., Okuno, S., and Fujisawa, H. (1997) J. Biochem. 122, 243–250
17. Sakagami, H., Saito, S., Kitani, T., Okuno, S., Fujisawa, H., and Kondo, H. (1998) Brain Res. Mol. Brain Res. 54, 311–315
18. Sakagami, H., Unemiyama, M., Saito, S., and Kondo, H. (2000) Eur. J. Neurosci. 12, 89–99
19. Tokumitsu, H., Enslin, H., and Soderling, T. R. (1995) J. Biol. Chem. 270, 19320–19324
20. Schulman, H. (1993) Curr. Opin. Cell Biol. 5, 247–253
21. Schulman, H., and Hanson, P. I. (1993) Neurochem. Res. 18, 65–77
22. Eto, K., Takahashi, N., Kinura, Y., Masuho, Y., Arai, K., Muramatsu, M. A., and Tokumitsu, H. (1999) J. Biol. Chem. 274, 22556–22562
23. Cruzelguez, F. H., and Means, A. R. (1993) J. Biol. Chem. 268, 26171–26178
24. Miranti, C. K., Ginty, D. D., Huang, G., Chatila, T., and Greenberg, M. E. (1995) Mol. Cell. Biol. 15, 3672–3684
25. Sun, P., Leu, L., and Mauer, R. A. (1996) J. Biol. Chem. 271, 3066–3073
26. Enslin, H., Tokumitsu, H., Stork, P. J., Davis, R. J., and Soderling, T. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10603–10608
27. Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) Nature 396, 584–587
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28. Hsu, L.-S., Tsou, A.-P., Chi, C.-W., Lee, C.-H., and Chen, J.-Y. (1998) J. Biomed. Sci. 5, 141–149
29. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, pp. 7.19–7.22, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
30. Chen, J.-Y., Funk, W. D., Wright, W. E., Shay, J. W., and Minna, J. D. (1993) Oncogene 8, 2159–2166
31. Tokumitsu, H., Takahashi, N., Eto, K., Yano, S., Soderling, T. R., and Muramatsu, M. (1999) J. Biol. Chem. 274, 15803–15810
32. Edwalds-Gilbert, G., Veraldi, K. L., and Milcarek, C. (1997) Nucleic Acids Res. 25, 2547–2561
33. Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S., and Evans, R. M. (1982) Nature 298, 240–244
34. Amara, S. G., Evans, R. M., and Rosenfeld, M. G. (1984) Mol. Cell. Biol. 4, 2151–2160
35. Crenshaw, E. B., Russo, A. F., Swanson, L. W., and Rosenfeld, M. G. (1987) Cell 49, 389–398
36. Rong Zeng, W., Soucie, E., Sung Moon, N., Martin-Soudant, N., Berube, G., Leduy, L., and Nepveu, A. (2000) Gene 241, 75–85
37. Acheson, N. H. (1984) Mol. Cell. Biol. 4, 722–729
38. Smith, C. W., Patton, J. G., and Nadal-Ginard, B. (1989) Annu. Rev. Genet. 23, 527–577
39. Naito, Y., Watanabe, Y., Yokokura, H., Sugita, R., Nishio, M., and Hidaka, H. (1997) J. Biol. Chem. 272, 32704–32708
40. Obhoko, S., Nishida, Y., Ryo, H., and Yamauchi, T. (1993) J. Biol. Chem. 268, 2052–2062
41. Loeth, O. P., de Lecea, L., Calbet, M., Danielson, P. E., Gautvik, V., Hovring, P. I., Walaas, S. I., and Gautvik, K. M. (2000) Brain Res. 869, 137–145
42. Tombes, R. M., and Krystal, G. W. (1997) Biochim. Biophys. Acta 1353, 281–292
43. Tombes, R. M., Mikkelsen, R. B., Jarvis, W. D., and Grant, S. (1999) Biochim. Biophys. Acta 1452, 1–11
44. Urquidi, V., and Ashcroft, S. J. (1995) FEBS Lett. 358, 23–26
45. Tokumitsu, H., Wayman, G. A., Muramatsu, M., and Soderling, T. R. (1997) Biochemistry 36, 12823–12827
46. Osawa, M., Tokumitsu, M. H., Swidells, M. B., Kurihara, H., Orita, M., Shibamura, T., Furuya, T., and Ikura, M. (1999) Nat. Struct. Biol. 6, 819–824
47. GuptaRoy, B., Beckingham, K., and Griffith, L. C. (1996) J. Biol. Chem. 271, 19846–19851
