Molecular Identification and Characterization of a Family of Kinases with Homology to Ca\(^{2+}\)/Calmodulin-dependent Protein Kinases I/IV*\(\dagger\)\(\ddagger\)

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Despite the critical importance of Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase (CaMK) II signaling in neuroplasticity, only a limited amount of work has so far been available regarding the presence and significance of another predominant CaMK subfamily, the CaMKI/CaMKIV family, in the central nervous system. We here searched for kinases with a core catalytic structure similar to CaMKI and CaMKIV. We isolated full-length cDNAs encoding three mouse CaMKI/CaMKIV-related kinases, CLICK-I (CL1)/doublecortin and CaM kinase-Like (DCAMKL)1, CLICK-II (CL2)/DCAMKL2, and CLICK-I/II-related (CLr)/DCAMKL3, the kinase domains of which had an intermediate homology not only to CaMKI/CaMKIV but also to CaMKII. Furthermore, CL1, CL2, and CLr were highly expressed in the central nervous system, in a neuron-specific fashion. CL1a and CL1b were shorter isoforms of DCAMKL1, which lacked the doublecortin-like domain (Dx). In contrast, CL2a and CL2b contained a full N-terminal Dx, whereas CLr only possessed a partial and dysfunctional Dx. Interestingly, despite a large similarity in the kinase domain, CL1/CL2/CLr had an impact on CRE-dependent gene expression distinct from that of the related CaMKI/CaMKIV and CaMKII. Furthermore, CL1, CL2, and CLr were highly expressed in the central nervous system, in a neuron-specific fashion. CL1a and CL1b were shorter isoforms of DCAMKL1, which lacked the doublecortin-like domain (Dx). In contrast, CL2a and CL2b contained a full N-terminal Dx, whereas CLr only possessed a partial and dysfunctional Dx. Interestingly, despite a large similarity in the kinase domain, CL1/CL2/CLr had an impact on CRE-dependent gene expression distinct from that of the related CaMKI/CaMKIV and CaMKII. Although these were previously shown to activate Ca\(^{2+}\)/cAMP-response element-binding protein (CREB)-dependent transcription, we here show that CL1 and CL2 were unable to significantly phosphorylate CREB Ser-133 and rather inhibited CREB-dependent transcription. We thus developed a dominant mechanism that bypassed CREB and was mediated by phosphorylated TORC2.

The availability of the human and other mammalian genome sequences provides a powerful means to dissect en masse the function of a particular class of proteins whose primary function can be deduced based on their primary sequences. The entire catalog of putative mammalian protein kinases, or kinome, has recently been established and curated (1, 2). This achievement per se is significant and now affords a better understanding of the biological role of kinases as regulatable switches under many physiologically and pathophysiologically critical circumstances. However, precise knowledge about the molecular characteristics of individual kinase molecules is still lacking (3).

As part of the large list of serine/threonine and tyrosine kinase families, the Ca\(^{2+}\)/calmodulin (CaM)\(^{3}\)-dependent protein kinase (CaMK) group stands out by the large number of its constituent kinases (1–3). Despite its nomenclature, however, only the classic CaMK subgroups such as CaMKII family, or the CaMKI/CaMKIV family, are genuinely catalytically Ca\(^{2+}\)/CaM-dependent. Most of the kinases of the CaMK group actually lack the characteristic Ca\(^{2+}\)/CaM-sensitive regulatory domain. They nonetheless belong to the CaMK group, because they share in common a significant homology in the primary structure of their kinase domains. Several classic CaMKs such as CaMKII and CaMKIV are highly expressed in the central nervous system and have been convincingly shown to play a critical role in long-term synaptic plasticity and in several forms of long-term memory (4–15). Thus, elucidation of the biological function of classic CaMKs is one of the key issues in neuroscience. Despite these insights, not many investigations have specific...

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\(\ddagger\) The abbreviations used are: CaM, calmodulin; CaMK, Ca\(^{2+}\)/calmodulin-dependent protein kinase; CLICK, CaMKI-like CREB-regulatory kinase candidate; DCAMKL, doublecortin and CaM kinase-Like; CREB, Ca\(^{2+}\)/cAMP-response element-binding protein; RACE, rapid amplification of cDNA end; ORF, open reading frame; PBS (−), Ca\(^{2+}\)/Mg\(^{2+}\)-free phosphate-buffered saline; CRE, Ca\(^{2+}\)/cAMP-response element; PKA, protein kinase A; SRE, serum-response element; IRES, internal ribosome entry sequence; EGFP, enhanced green fluorescent protein; CBP, CREB-binding protein; DA, dominant active; WT, wild-type; EST, expressed sequence tag; indel, insertion and deletion polymorphism; DCK, doublecortin-like kinase; DCLK, doublecortin-like kinase; cpg, candidate plasticity-related gene; Dx, doublecortin-like domain; KID, kinase-inducible domain; TORC, transducer of regulated CREB activity; ANOVA, analysis of variance; aa, amino acid(s).

\(\ddagger\) The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AY968047, AY968048, AY968049, AY968050, AY968051, and DQ286388.

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cally addressed how many CaMKs or related kinases are expressed in the central nervous system, and how many of them actually might play a role in regulation of brain circuitry formation, maturation, or plasticity. For example, it is only recently that the diversity of the CaMKI subfamily consisting of four separate genes has been appreciated (1, 2, 16–19) and that several intriguing novel functions have been proposed for neuronal CaMKI (20–22). To better understand the complexity of neuronal CaMK profile, we here have carried out a degenerate PCR-based search for kinases with a core catalytic structure similar to CaMKI and CaMKIV. Consistent with our goal, we identified three mouse CaMKI/CaMKIV-related kinases, CLICK-I (CL1)/DCAMKL1, CLICK-II (CL2)/DCAMKL2, and CLICK-I,II-related (CLr)/DCAMKL3, the kinase domains of which were structurally related not only to CaMKIV and CaMKI but that also had comparable homology to CaMKII. Furthermore, CL1, CL2, and CLr were highly expressed throughout the central nervous system, in a neuron-specific fashion, from embryonic stages until adulthood. We identified several distinct open reading frames for CL1, CL2, and CLr. Whereas CL1α and CL1β are shorter isoforms of DCAMKL1 that lack the doublecortin-like domain (23–30), CL2α and CL2β contain an N-terminal doublecortin-like domain (Dx), which, via tight association with the microtubules, allowed specific dendritic localization in mature hippocampal neurons. In contrast, CLr only contained an incomplete Dx-like homology and was unable to localize with microtubules. Interestingly, despite a large structural similarity in the kinase domain, the functional impact of kinase activity of CL1/CL2/CLr kinases appeared distinct from the related CaMKI/CaMKIV and CaMKII. Indeed, although both CaMKI/CaMKIV and CaMKII branches of the CaMK family are established CaMKII/CaMKIV-dependent CREB-regulatory kinase candidates (CLICKs), and named CLICK-I (CL1) and CLICK-II (CL2), respectively. EST search revealed the presence of mouse orthologs for both CL1 and CL2, but none represented full-length cDNAs. Full-length 5′-ends and 3′-ends for mouse CL1 and CL2 were identified by rapid amplification of 5′- and 3′-cDNA ends (5′- and 3′-RACE) procedures using a Smart RACE cDNA amplification kit (Clontech). We obtained multiple 3′-RACE fragments of different sizes for both CL1 and CL2, and sequencing confirmed the presence of at least two C-terminal splice variants in each case. As for the 5′-end, the most remote ATG codon that was found in-frame was considered as the putative initiation site of the open reading frame. Finally, to isolate genuine full-length open reading frames (ORFs), PCR was carried out between the putative initiation ATG and the two alternate stop codons using Advantage High Fidelity Polymerase (Clontech) and ICR mouse adult hippocampal cDNA as a template. A CL1- and CL2-related mouse FANTOM clone C730036H08 was obtained from Dnaform (Tokyo, Japan).

EXPERIMENTAL PROCEDURES

All reagents were of analytical grade, and all experiments involving radioisotopes, animals, or recombinant DNAs were approved by recombinant DNA and Animal Research Committees and carried out following guidelines established at the University of Tokyo and Kyoto University, in accordance with regulations and laws set by the Japanese government.

Molecular Cloning of Full-length CLICK-I, CLICK-II, and CLICK-I,II-related cDNAs—To screen for the presence of yet uncharacterized CaMK-like kinases, degenerate oligonucleotide primers were constructed as follows: CaMK5′-1, 5′-GTICAYMIGIYMTCAARC-3′ (Y = T/C, M = A/C, R = A/G); CaMK5′-2, 5′-GTICAYMGIMYMTGARCC-3′ (Y = T/C, M = A/C, R = A/G); CaMK5′-3, 5′-GTICAYMIGIYMTCAARC-3′ (Y = T/C, M = A/C, R = A/G); and CaMK3′, 5′-CCYGGRGTYCCACAIYCGT-3′ (Y = T/C, R = A/G).

100 pmol of a 1:1:1 mixture of CaMK5′-1/CaMK5′-2/CaMK5′-3 and 100 pmol of CaMK3′ were included with ~100 ng of Sprague-Dawley rat adult hippocampal cDNA in a standard 50-μl PCR carried out for 35 cycles at 30-s denaturation at 94 °C, 1-min annealing at 48 °C, 1-min extension at 72 °C, using native Taq polymerase (Genetaq, Nippon Gene). After confirming the presence of an expected ~140-bp band, this PCR fragment was co-digested with BspHI, BstXI, BclI in NEB buffer 2 (50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.9, New England Biolabs), because these three enzymes were expected to digest the PCR fragments amplified from known CaMKI/CaMKIV cDNAs. A portion of the ~140-bp fragment consistently remained resistant to enzyme digestion. To identify this fragment, this resistant PCR band was gel-purified from a 3% NuSieve GTG-agarose gel, and subcloned into T7Blue-2 (Novagen) by TA cloning. Isolation of independent colonies and sequence analysis revealed the presence of two related inserts that were homologous to, but had only limited identity with, known members of the CaMKI/CaMKIV family. These fragments were tentatively considered as CaMK-like CREB regulatory kinase candidates (CLICKs), and named CLICK-I (CL1) and CLICK-II (CL2), respectively. EST search revealed the presence of mouse orthologs for both CL1 and CL2, but none represented full-length cDNAs. Full-length 5′-ends and 3′-ends for mouse CL1 and CL2 were identified by rapid amplification of 5′- and 3′-cDNA end (5′- and 3′-RACE) procedures using a Smart RACE cDNA amplification kit (Clontech). We obtained multiple 3′-RACE fragments of different sizes for both CL1 and CL2, and sequencing confirmed the presence of at least two C-terminal splice variants in each case. As for the 5′-end, the most remote ATG codon that was found in-frame was considered as the putative initiation site of the open reading frame. Finally, to isolate genuine full-length open reading frames (ORFs), PCR was carried out between the putative initiation ATG and the two alternate stop codons using Advantage High Fidelity Polymerase (Clontech) and ICR mouse adult hippocampal cDNA as a template. A CL1- and CL2-related mouse FANTOM clone C730036H08 was obtained from Dnaform (Tokyo, Japan).

Although this clone was deposited as a partial cDNA, full sequencing of its 5′-end revealed a C insertion at position 566 of the originally deposited sequence. Correction of this frameshift unmasked a previously unrecognized cryptic N terminus with a functional ATG initiation codon starting from position 514. In total, six distinct full-length cDNAs were obtained in this study and designated as CLICK-1α (GenBank™ accession number AY968047), CLICK-1β (AY968048), CLICK-1αα (AY968049), CLICK-1β1 (AY968050), CLICK-1β2 (AY968051), and CLICK-1,II-related (DQ286388).

Genomic Mapping—Kinase domain sequences of CLICK-I (CL1), CLICK-II (CL2), and CLICK-I,II-related (CLr) were mapped on the Human and Mouse Genome Database, as made available by NCBI and Celera. BAC clones mapped to 3D and 3F1 were obtained from the BACPAC Resource Center (Children’s Hospital Oakland Research Institute, Oakland, CA) to ascertain that both CL1/Deamk1I and CL2/Deamk2I loci were
located indeed in relative vicinity on the mouse chromosomes 3D and 3F1, respectively.

**Kinase Characterization**—All expression vectors for wild-type and mutant CL1, CL2, and CLr were constructed in either pDEST26 or pEGFP-C1 vector backbones using PCR, TOPO cloning or GATEWAY technology (Invitrogen), with addition of either N-terminal Myc tag, HA tag, or EGFP tag, as indicated. Domain deletion and introduction of point mutations were carried out on CL1α and CL2β backbones, unless stated otherwise, using PCR and by use of QuikChange Mutagenesis kit (Stratagene). The presence of proper inserts and mutagenesis was confirmed by DNA sequencing. The plasmids used in this report were as follows: CL1α (amino acid (aa) position 1–421), CL1αKD (aa 1–421 with Lys at position 111 mutated to Ala), CL1ΔC (aa 1–353), CL2α (aa 1–771), CL2β (aa 1–714 of CL2β2), ΔΔx-CL2β (aa 377–714 of CL2β2), ΔΔx-CL2βKD (aa 377–714 with Lys at position 437 mutated to Ala), ΔΔx-CL2ΔC (aa 377–679 of CL2β2), and CL2-Dx (aa 1–431 of CL2α). These constructs were transfected in COS-7 cells, and crude lysates were prepared in a Ca2+-free buffer containing (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 1 mM dithiothreitol, 25 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.1 mM sodium vanadate, 1 mM EGTA, 0.1 μM calcinulin A, and 1 × EDTA-free Complete protease inhibitors (Roche Applied Science). Immunoprecipitates were obtained using an anti-Myc monoclonal antibody (Santa Cruz Biotechnology) and were subjected to kinase assays as described previously (17) except that Ca2+ and CaM were removed from the kinase reaction buffer. Integrity of expressed proteins was verified by silver staining or by immunoblot using a mouse anti-Myc monoclonal antibody (Santa Cruz Biotechnology). The 32P incorporation was visualized using BioMax x-ray films (Eastman Kodak). The presence of proper inserts and mutagenesis were as follows: CL1, CL2, and CLr were constructed in either pBluescript II KS+ vector backbones, unless stated otherwise, using PCR and by use of QuikChange Mutagenesis kit (Stratagene). The presence of proper inserts and mutagenesis were confirmed by DNA sequencing. The plasmids used in this report were as follows: CL1α (amino acid (aa) position 1–421), CL1αKD (aa 1–421 with Lys at position 111 mutated to Ala), CL1ΔC (aa 1–353), CL2α (aa 1–771), CL2β (aa 1–714 of CL2β2), ΔΔx-CL2β (aa 377–714 of CL2β2), ΔΔx-CL2βKD (aa 377–714 with Lys at position 437 mutated to Ala), ΔΔx-CL2ΔC (aa 377–679 of CL2β2), and CL2-Dx (aa 1–431 of CL2α). These constructs were transfected in COS-7 cells, and crude lysates were prepared in a Ca2+-free buffer containing (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 1 mM dithiothreitol, 25 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.1 mM sodium vanadate, 1 mM EGTA, 0.1 μM calcinulin A, and 1 × EDTA-free Complete protease inhibitors (Roche Applied Science). Immunoprecipitates were obtained using an anti-Myc monoclonal antibody (Santa Cruz Biotechnology) and were subjected to kinase assays as described previously (17), except that Ca2+ and CaM were removed from the kinase reaction buffer. Integrity of expressed proteins was verified by silver staining or by immunoblot using a mouse anti-Myc monoclonal antibody (Santa Cruz Biotechnology). The 32P incorporation was visualized using BioMax x-ray films (Eastman Kodak Co.). Pull-down assays using CaM beads were done as described previously (17).

**Transcript Analyses**—Northern blot analyses were carried out using pre-made poly(A)+ RNA blots (2 μg per lane) obtained from Clontech. Blots were hybridized using a 32P-labeled DNA probe corresponding to the nucleotide position 58–1116 of CL1α (AY968047), 3–2039 of CL2α (AY968049), and 514–2373 of CLR (DQ286388) (for position see Fig. 1A), according to Ref. 17. In situ hybridization using DIG technology (Roche Diagnostics) was performed essentially as described (38) with some modifications. In brief, anesthetized mice were perfusion-fixed with Tissue Fixative (GenoStaff, Inc.), and dissected tissues were sectioned after paraffin embedding. For generation of antisense and sense cRNA probes, 294–, 255–, and 1860-bp fragments corresponding to the nucleotide position 898–1191 of CL1α, 860–1114 of CL2α, and 514–2373 of CLR were subcloned into pBluescript II KS+ vector (Stratagene). Digoxigenin-labeled cRNA probes were prepared with DIG RNA labeling Mix (Roche Applied Science). Coloring reactions were performed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, an alkaline phosphatase color substrate, and tissue sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals Co., Ltd.). After mounting, 24-bit color images were acquired by scanning the sections using an Epson GT-8400UF digital scanner at 3200-dpi resolution (Fig. 3C and supplemental Fig. S1). DIG signals were isolated by uniformly subtracting the counterstaining color component using Photoshop version 7.0.1 (Adobe) and displayed in 8-bit grayscale without further correction (Fig. 3, B and D).

**Immunocytochemical Analysis of CL1 and CL2 Expressed in HeLa Cells and in Central Neurons**—Cerebelli and hippocampi were obtained from postnatal day 0–1 ICR mice, and cerebellar granule neurons and hippocampal neurons were cultured as described before (39, 40). Plasmid transfection was carried out using Lipofectamine 2000 (Invitrogen), at 0 day in vitro for cerebellar granule cells or at 9 days in vitro for hippocampal neurons, essentially as described (17, 41). About 48 h after transfection, HeLa cells and neurons were fixed in 4% paraformaldehyde/4% sucrose/Ca2+/Mg2+-free phosphate-buffered saline (PBS(−)) at 37 °C for 15 min, washed with 0.1 M glycine/PBS(−) at room temperature. Blocking and permeabilization was carried out as described (11). Primary and secondary antibody reactions were performed overnight at 4 °C and 1 h at room temperature, respectively. Antibodies used were: rabbit anti-Myc polyclonal (Santa Cruz Biotechnology), mouse anti-β-tubulin monoclonal (Sigma), and Alexa488- or Alexa555-conjugated anti-rabbit or anti-mouse antibodies (Molecular Probes). Immunofluorescent images were acquired using an Olympus DP-70 charge-coupled device camera (Fig. 4) or a Zeiss LSM510META confocal microscope (Supplemental Fig. S2). For the latter, maximal projection images were obtained from z-stacks of 10–15 confocal sections covering the entire depth of cells within the field of view.

**Luciferase Assay**—Dual luciferase assay was carried out in COS-7 cells essentially as described (17), except that 24-well plates were used for assays and luciferase activities were monitored using a Fluoroskan Ascent FL plate reader (ThermoLab systems), with EF1-Rluc as a control vector (42). CRE-luc, Gal4-CREB, UAS-luc, and PKACat constructs were from Stratagene. An CRE-luc (3D.Aluc) vector was obtained from Richard Treisman (Imperial Cancer Research Foundation). A V14Rho expression vector was used as described (39). A lacZ expression vector was created as a mock control in the pDEST26 backbone, based on SinRep/LacZ (Invitrogen). CaMK4ΔC and CaMK2ΔC were constructed in pRES-EGFP vectors (Clontech) as described (43), based on CaMK4DA and CaMK2DA vectors (33) kindly provided by Richard Maurer (Oregon Health and Sciences University). A full-length CBP cDNA (44) was obtained from Akiyoshi Fukushima (Tsukuba University), with permission from Marc Montminy (Salk Institute) and subcloned in frame immediately downstream of Gal4-(1–147) in a mammalian Matchmaker two-hybrid vector pM (Clontech) to yield a Gal4-CBP vector. To construct TORC2-GFP, an open reading frame of human TORC2 (OriGene clone TC107319) was subcloned by PCR into the EcoRI/Sall sites of pEGFP-C1 (Clontech). All point mutants were constructed using PCR and the QuikChange mutagenesis kit (Stratagene). Gal4-CREB-DA (pRc/RSV-GAL4-CREBpHIELM) and Gal4-CREB-WT (pRc/RSV-GAL4-CREB) (45) were kind gifts of Richard Goodman (Vollum Institute), and EF1-Rluc was kindly provided by Akiko Tabuchi and Masaaki Tsuda (Toyama Medical and Pharmaceutical University).
RESULTS

Molecular Cloning of CaMK-like Kinases Based on Sequence Similarity around the Conserved Catalytic Domain and Activation Loop of CaMKI/CaMKIV—During a screen for potential CREB-regulatory kinases, we sought for key determinants within the kinase domain of known in vitro CREB kinases such as CaMKI (17, 46, 47) and CaMKIV (11, 33, 34). Sequence analysis revealed a very high degree of conservation between CaMKI and CaMKIV in two motifs HRDLK-PENLL (aa 154–163 in rat CaMKIV) and CGTPGY (aa 194–199), critical for kinase catalytic activity and CaMKK-dependent activation, respectively (35–37). We therefore designed degenerate oligonucleotide primers based upon these conserved motifs and, by PCR using cDNA reverse-transcribed from Sprague-Dawley rat adult hippocampal poly(A)^+ RNA, we attempted to probe for the presence of yet uncharacterized CaMK-like species. A PCR-fragment of 140 bp was detected, consistent with the expected size of the amplified product from cDNAs of either CaMKI, CaMKIV, or CaMKIV. Based on available GenBank™ information, these predicted PCR products would be cleaved by BspHI, BstXI, or BclI. A diagnostic restriction enzyme reaction with these three enzymes, however, revealed that a previously unknown species, resistant to all three enzyme digestions, was present. To identify this molecular species, we gel-purified this digestion-resistant 140-bp fragment and subcloned it into the T7Blue-2 vector. Random sequencing of 78 independent clones revealed that 73 clones encoded a kinase or a kinase-like gene, and among them, more than 60 clones represented kinases belonging to the large CaMK group (1), indicating that the kinase screen was carried out properly. Three major species was isolated more than 10 times (49 times in total), and among them, two were yet uncharacterized, homologous to each

![FIGURE 1.](https://example.com/figure1.png)

**Characterization of Novel Kinases with Homology to CAMK I/IV**

![A](https://example.com/figureA.png)

**Figure A**. Schematic structure of cloned open reading frames for mouse CL1, CL2, and CLr cDNAs. The two isolated forms of CL1, CL1α and CL1β, were mouse orthologs for KIAA0369-B5/CaMKII/DCLK-short-B/DCK-2. CL1α and CL1β, shared a common N terminus but had distinct C termini, respectively. CL1β contained an insertion of Glu at position 68 and of Gin at position 96, respectively, likely reflecting insertion and deletion polymorphisms. A homologous kinase domain (hatched) was shown, and CL2 contained an N-terminal doublecortin-like domain (Dx) with two repeated motifs (dark-shaded), homologous to those present in a longer CL1 isoform DcAMKL1. CLr only contained a partial Dx with one motif with a low identity score. The borders of each domain are numbered by the amino acid position within the ORF. The horizontal lines represent the fragments originally isolated by degenerate PCR (thick lines), and fragments used to generate in situ hybridization probes (solid lines) or Northern blot probes (thin lines) in Fig. 3. B, exon-intron structure of mouse CL2 and CLr genes. Comparison of mouse genomic sequence with the isoforms of CL2 and CLr cDNAs revealed that the mouse CL2 and CLr genes contained at least 18 and 5 exons, respectively. In comparison with CL2β, CL2α differed by the skipping of exon 17, while in CL2β, an insertion of three nucleotides occurred at the 5'-end of exon 7 by an alternative splice acceptor usage. C, mouse CL1 and CL2 genes were both located on mouse chromosome 3, whereas CLr gene was on mouse chromosome 9 (left), and human counterpart genes were on human chromosomes 13, 4, and 3, respectively (right).
other and were relatively related to the kinase domains of CaMKI and CaMKIV. These fragments were considered as partial clones from CaMK-like CREB regulatory kinase candidates (CLICKs), which were designated CLICK-1 (CL1) and CLICK-II (CL2), respectively (Fig. 1A, thick lines). Using EST search and sequential RACE techniques, full-length mouse cDNAs were then obtained for both CL1 and CL2. Two distinct ORFs were isolated for CL1 (CL1α, 421 aa and CL1β, 433 aa (Fig. 1A)) and three distinct ORFs were isolated for CL2 (CL2α, 771 aa; CL2β1 715 aa; and CL2β2, 714 aa (Fig. 1, A and B)). CL1β and CL2β1 contained an insertion of Gln at position 68 and of Gln at position 368, respectively, likely reflecting insertion and deletion polymorphisms (indels). Examination of genomic exon-intron boundaries revealed that CL2β1 and CL2β2 were borne out of use of distinct splice acceptor sites at the 5′-terminal end of exon 7 and resulted in an addition of one extra amino acid in CL2β1 (Fig. 1B).

During the course of our study, CL1α and CL1β were found to encode mouse orthologs for KIAA0369-BS/CaMKVI/DCLK-short-B/DCK-β1 (24, 28, 48, 49) and cpg-16/KIAA0369-BL/DCLK-short-A/DLK-β2 (24–26, 48, 49), respectively. A longer splice variant possessing an N-terminal doublecortin-like domain (Dx), DCLK/CAMKL1 (23, 24, 27, 29, 30), was further reported to be highly present in the embryonic brain (50–52). However, the precise biological function for DCAMKL1 and its numerous splice variants has not been established. In contrast to CL1/DCAMKL1, for which many cloning efforts, including ours, revealed the presence of shorter isoforms in the adult brain, all CL2 transcripts isolated from adult mouse hippocampal cDNAs in this study contained the Dx-like domain in their open reading frames, and no shorter splice variants were found. This indicated that CL2 may be a predominant Dx-containing CL/DCAMKL isoform in the mature central nervous system. Interestingly, genomic mapping revealed that the genomic loci for CL1 and CL2 genes were both located on mouse chromosome 3 (3D and 3F1, respectively) within close proximity and in an opposite tandem direction, suggesting that these two genes originated from gene duplication during evolution (Fig. 1C). In human, both genes were separated on chromosomes 13 (13q13) and 4 (4q31.23), respectively (Fig. 1C).

Rsequencing of a CL1/2-related cDNA, originally reported as a partial cDNA (FANTOM clone C730036H08), revealed a yet unreported nucleotide insertion within its putative open reading frame. The correction of this frameshift revealed the presence of a previously unrecognized N-terminal end with an in-frame ATG initiation codon. The accuracy of the newly obtained cDNA sequence, designated as CLICK-I,II-related (CLr), was confirmed by a gapless sequence alignment to existing mouse genome sequences. Furthermore, a GFP fusion protein constructed by a single round of PCR subcloning yielded a recombinant protein of the expected size as measured by SDS-PAGE and by Western blot (data not shown). Exon-intron structures were, however, distinct from CL1/DCamkl1 and CL2/Dcamkl2 genes (Fig. 1B), and the mouse CLr/Dcamkl3 gene was mapped to chromosome 9 (9F3, Fig. 1C).

CL1/DCAMKL1, CL2/DCAMKL2, and CLr/DCAMKL3 Form a Novel Kinase Subfamily with Intermediate Homology with CaMKI/CaMKIV and CaMKII—Comparison of kinase domain sequence identity revealed that CLr/DCAMKL3 was indeed the closest kinase to CL1 and CL2 (56 and 54% identity, respectively), followed by CaMKIV (44 and 42%) and CaMKII (43 and 42%) (Fig. 2). Furthermore, using ClustalW, the CL1/CL2/CLr/DCAMKL kinase subfamily was confirmed to actually lie between the CaMKII gene family and the CaMKI/CaMKIV gene family. This raised the possibility that the kinase activity of CL1, CL2, and CLr may be regulated in a manner similar to CaMKI/ CaMKIV and CaMKII. C-terminal deletion mutants for both CL1 (CL1.ΔC) and CL2 (ΔDx-CL2.ΔC) had a significant amount of Ca2+/CaM-independent kinase activity (data not shown), in keeping with prior findings obtained for CaMKII and CaMKIV (35–37). Surprisingly, however, intact forms of both CL1 and CL2 also showed notable amounts of kinase activities, even in the absence of calcium, and lacked significant CaM binding (data not shown), consistent with prior reports (26, 48, 53). Taken together, CL1/DCAMKL1, CL2/DCAMKL2, and CLr/DCAMKL3 are likely to form a novel subfamily of kinases with intermediate homology with CaMKI/CaMKIV and CaMKII, but with significantly reduced Ca2+/CaM affinity and dependence.

CL1, CL2, and CLr Transcripts Have Broad and Overlapping Distribution Patterns in the Adult Forebrain, but CL1 and CL2 Are Predominant in the Embryonic Central Nervous System—We next examined mRNA expression of CL1/DCAMKL1, CL2/DCAMKL2, and CLr/DCAMKL3, both in adults and in embryonic mouse tissues. Northern blot analysis revealed an essentially brain-specific expression pattern for both CL1 and CL2, but CLr was expressed in several peripheral organs such as liver and kidney (Fig. 3A). At least five, four, and two distinct mRNA
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species were detected for CL1, CL2, and CLr, respectively. A substantial amount of CL1 and CL2 mRNA was expressed from embryonic day 11 (E11) on, throughout development of the central nervous system until the brain reaches full maturity (Fig. 3A). CLr mRNA, however, was hardly detected in embryos (Fig. 3A). In situ hybridization of adult mouse brain sections confirmed expression of CL1, CL2, and, to a lesser extent, CLr mRNA in neurons, but not in glial cells, in most forebrain areas (Fig. 3B and supplemental Fig. S1A). In adult mice, expression of CL1, CL2, and CLr was particularly evident in the hippocampal CA1 pyramidal cell layer (Fig. 3C). At embryonic stages, specific CL1 and CL2 mRNA hybridization signals were predominantly detected in the central nervous system, throughout the forebrain, midbrain, hindbrain, and the spinal cord, with indistinguishable distribution; however, specific CLr mRNA signal was barely detectable (Fig. 3D and supplemental Fig. 1B).

Association to Microtubules via a Doublecortin-like Domain in CL2 but Not in CLr—Previous studies on doublecortin and DCAMKL1 have shown the critical role of doublecortin-like domain (Dx) in microtubule attachment (51, 52, 54). To test the role of Dx in CL2, we transfected wild-type and mutant CL2 constructs in HeLa cells and examined whether they co-localized to microtubules and how they affected microtubule organization. Full-length CL2 (CL2β), a kinase-dead full-length CL2 (CL2βKD), and the doublecortin-like domain only of CL2 (CL2-Dx) all co-localized with microtubule structures, whereas Dx removal (∆Dx-CL2β) resulted in a diffuse cytosolic distribution (Fig. 4A). Heavy bundling of microtubules was observed in a significant proportion of cells expressing CL2 constructs containing Dx such as CL2β, CL2βKD, and CL2-Dx, but was absent in cells transfected with a CL2 mutant lacking Dx (∆Dx-CL2β) (Fig. 4B). Together, the presence of Dx in CL2 appeared to be necessary and sufficient for CL2 co-localization with microtubules in HeLa cells (Fig. 4A), and the critical importance of Dx was also shown in cultured postmitotic neurons such as hippocampal pyramidal and cerebellar granule neurons (Ref. 53 and supplemental Fig. S2, A and B). Interestingly, CLr localized diffusely in the cytoplasm and in the nucleus with no particular association with the microtubule structures in HeLa cells, indicating that the weakly homologous Dx-like domain in its N terminus (Fig. 1A) may not be sufficient to provide efficient microtubule binding (Fig. 4C). In dividing HeLa cells, overexpression of Dx-containing CL2 constructs induced severe cytokinesis phenotypes such as aberrant multinucleate cells (>10% of transfected cells versus a few percent in mock controls). This phenotype was completely abolished by the removal of Dx and was attenuated in the absence of kinase activity in Dx-containing CL2 mutants (Fig. 4, D and E). This indicated that the intrinsic activity of an overexpressed Dx domain, or alternatively, sequestration of binding sites for endogenous Dx domains severely interfered with dynamics of endogenous microtubule structures and suggested the possibility that CL2 kinase activity may modulate to some extent the interaction of Dx with the microtubules.

The Kinase Activity of CL1/CL2/CLr Targets Components of CREB-dependent Gene Expression in a Manner Distinct from That of CaMkI, CaMkIV, and CaMkII—Finally, we addressed whether the kinase activity of CL1/CL2/CLr had an impact to gene transcription that was similar or distinct from that of CaMkI/CaMkIV and CaMkII. Previous work, including ours, has established that activated forms of CaMkI, CaMkIV, and CaMkII all have significant effects on CRE-mediated gene expression (11, 17, 31–37, 46, 47). Kinase-domain-only constructs (CL1 and ∆Dx-CL2, -WT, and -KD) were transfected into COS-7 cells along with CRE-luc and a control Renilla-luc vector driven by the EF1 promoter as a control, and the Luc/Renilla ratio was calculated as an index for CRE-dependent gene expression. Expression of an increasing amount of wild-type kinase constructs gradually reduced CRE-dependent gene expression in forskolin-stimulated COS-7 cells, whereas kinase-dead constructs showed no decrease at all (Fig. 5A). This clearly indicated that the kinase activity in CL1 and CL2 was sufficient for suppressing forskolin-stimulated CRE-dependent gene expression. These results were replicated using C-terminal-deleted kinases (CL1ΔC and ∆Dx-CL2ΔC) as well (Fig. 5B and data not shown), and this CL1/CL2-mediated repression of CRE-dependent gene expression was rather specific, because Rho-stimulated SRE-dependent gene expression (55) was completely unaffected (Fig. 5B). Such specificity would be easily accounted for if CL1 and CL2 were able to phosphorylate and modulate specific regulators involved in CRE-dependent transcription. One obvious candidate is CREB, which has repeatedly been demonstrated to be phosphorylated at its Ser-133 residue by activated CaMkI, CaMkIV, and CaMkII (11, 17, 31–37, 46, 47). However, despite the structural similarity in the kinase domain with CaMkI, CaMkIV, and CaMkII, CL1 and CL2 strongly repressed both CaMkI- and CaMkIV-induced (Fig. 5C, left panel, CaMk4ΔC) or PKA-induced CREB activation as tested using a Gal4-CREB/US-Luc system (Fig. 5C, right panel, PKAcat). Thus, in contrast to CaMkI and CaMkIV, CL1 and CL2 clearly were not able to stimulate CREB. Rather, CL1 and CL2 tended to inhibit CREB-dependent transcription. Intrigu-
ingly, this CL phenotype could be qualitatively replicated by expression of a constitutively active form of CaMKII (CaMK2\(H_9004\)/H9004C) (Fig. 5C).

Repression of CREB-mediated transcription, as measured by a Gal4-CREB assay, could occur, in principle, by several mechanisms. Two evident possibilities are the inhibition of CREB
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Ser-133 phosphorylation machinery, and the stimulation of the reverse process, i.e. dephosphorylation of phospho-CREB. If either of these two mechanisms were involved, one would predict that the CL1/CL2 effect would be significantly attenuated by a shunting manipulation, such as expression of a dominant active form of CREB (CREB-DA) that possessed a modified kinase-inducible domain with a DIEDML mutation that mimicked an Ser-133-phosphorylated state and that constitutively bound to CBP with high affinity in the absence of any phosphorylation event (45). However, contrary to this expectation, CL-mediated CREB repression remained significant even when CREB activity was constitutively elevated (Fig. 6A). Thus, the CL-mediated inhibitory effect was dominant and did not result from preventing CREB activation by Ser-133 phosphorylation, or by stimulating phospho-CREB dephosphorylation.

An alternative explanation would be to consider an inhibitory phosphorylation event on CREB: indeed, phosphorylation of Ser-142 of CREB by CaMKII (33, 46) was previously shown to mediate an inhibitory CRE response, at least in a heterologous system (33, 46). In keeping with such a possibility, we had observed a qualitatively similar degree of inhibition of CRE-dependent gene expression by introducing an active CaMKII (CaMK2ΔC) (Fig. 5C). To directly test whether Ser-142 had any role in the inhibitory effect mediated by CL1/CL2, we introduced a Ser-142 → Ala mutation into the Gal4-CREB construct and examined its consequence on CL-mediated CREB inhibition. Again, however, both CL1ΔC and ΔDx-CL2ΔC were still able to significantly inhibit forskolin-stimulated CREB-dependent transcription, which argued that Ser-142 phosphorylation was unlikely to be the major mechanism of this repression (Fig. 6B, upper and lower panels). This is in sharp contrast to the case of CaMKII, which was previously shown to activate, rather than inhibit, Gal4-CREB/S142A-dependent transcription (33, 46).

An additional possibility would be that CL1 and CL2 may alter the function of the CREB target CBP, as has been suggested for CaMKIV (56–58). We tested this by using a Gal4-CBP construct. Consistent with prior reports, both CaMKIV and PKA were able to significantly augment CBP-mediated transcription (Fig. 6C). However, CL1ΔC and ΔDx-CL2ΔC revealed no such activity, and no consistent inhibitory activity was shown either (Fig. 6C). Thus, unlike CaMKIV, neither CL1 nor CL2 appeared to modulate CBP significantly.

Recent evidence suggested that a novel type of CREB coactivator, transducer of regulated CREB activity (TORC), may mediate a significant part of CREB-dependent gene expression (59, 60). In particular, a critical phosphorylation site was found at serine-171 of TORC2, one of the TORC isoforms, and control of its phosphorylation state by either a serum-inducible kinase or an adenosine-monophosphate-dependent protein kinase was shown to regulate nucleocytoplasmic shuttling of TORC. Thus, increase in phospho-Ser-171-TORC2 prevented CREB activation by Ser-133 phosphorylation, as illustrated in Fig. 6D. We next examined whether CL1/CL2 mediated a similar effect on CREB, and found that both CL1ΔC and ΔDx-CL2ΔC were able to strongly inhibit CREB-mediated transcription (Fig. 6E). This is consistent with a role for Ser-171 phosphorylation in the repression of CREB activity by CL1/CL2. However, unlike CaMKIV, neither CL1 nor CL2 appeared to modulate CBP significantly.

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**DISCUSSION**

Herein, we report the isolation and characterization of mouse cDNAs encoding three related kinases, CL1/DCAMKL1, CL2/DCAMKL2, and CLr/DCAMKL3, whose kinase domain revealed significant homology with that of CaMKI and CaMKIV. CL1α and CL1β were mouse orthologs of shorter isoforms of DCAMKL1 that did not contain a doublecortin-like domain (Dx). All isolated isoforms of CL2 (CL2α, CL2β1, and CL2β2), however, included an intact microtubule-binding Dx at its N terminus, whereas CLr only contained a partial and dysfunctional Dx. Interestingly, the mouse Dcamkl1 and Dcamkl2 genes were located within relative proximity on an identical chromosome 3 in the mouse genome, in opposite direction, whereas the third related gene, Dcamkl3, has been mapped to mouse chromosome 9. Surprisingly, a dendrogram analysis, examining a putative phylogenetic relationship based on the primary structure of the kinase domain, revealed that, in fact, the kinase domains of all three DCAMKL genes formed a novel kinase group that had an intermediate homology with both the CaMKI/CaMKIV and CaMKII subfamilies of CaMK. Given that CL1/DCAMKL1, CL2/DCAMKL2, and CLr/DCAMKL3 also share similarity with CaMKI/CaMKII/CaMKIV in their neuron-specific expression in the adult brain, it is likely that the DCAMKL genes, CaMKI/CaMKIV subfamily, and CaMKII subfamily may have co-evolved from a common ancestral CaMK during evolution.

Recently, the role of doublecortin in formation of forebrain layer structures has been extensively characterized (e.g. Ref. 54). It is now believed that doublecortin largely functions as a microtubule-associated protein via its privileged interaction with a microtubule-binding domain.

FIGURE 6. Dominant suppression of CREB-mediated transcription by a mechanism that bypasses CREB. A, CL1/CL2-mediated suppression occurred to a similar extent on a non-phosphorylatable, constitutive active form of CREB (DA, right panel) as on a PKA-activated wild-type CREB (WT, left panel). This indicated that this suppression was independent of the phosphorylation status of stimulatory Ser-133 site on CREB. Thus, neither CL1 nor CL2 were likely to act by inhibition of the CREB Ser-133 phosphorylation machinery or by stimulation of phospho-Ser-133 dephosphorylation. **, p < 0.01 (one-way ANOVA with Tukey post hoc test). B, CL1 (upper panel)/CL2 (lower panel)-mediated suppression occurred to a similar extent even on a mutant CREB, in which Ser-142, the previously characterized CaMKII-targeted inhibitory site, was replaced with Ala. Consistent with these experiments, in vitro phosphorylation of recombinant CREB was below the limit of detection (data not shown). Together, our data suggested that CL1/CL2 kinase activities act by a mechanism that bypasses CREB. **, p < 0.01; ***, p < 0.001 (two-tailed Student’s t test). C, CL1/CL2 effect on CBP-mediated transcription is again distinct from the CaMKIV/PKA-mediated stimulation of CBP-dependent transcription. Each data point on the graph represents mean ± S.E. calculated from triplicates. The results shown are representative of multiple independent sets of experiments. *, p < 0.05; ***, p < 0.001 (one-way ANOVA with Tukey post hoc test).
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FIGURE 7. CL2-induced suppression of CREB activity is mediated by a phosphor-Ser-171-TORC2-dependent mechanism. A, kinase activity of CL2 favored nuclear export/cytoplasmic retention of TORC2. Under our basal culture condition, TORC2-GFP was found to be largely concentrated in the nucleus of COS-7 cells. However, co-expression of an active form of CL2 (∆Dx-CL2ΔC) potently inhibited nuclear import of TORC2-GFP and increased its cytoplasmic content (arrowheads). Scale bar, 20 μm. B, monitoring of total fluorescence values of TORC2-GFP revealed a significant transfer of TORC2 protein from the nucleus to the cytosol in a CL2-dependent manner, as measured by a shift in cytosol/nucleus ratio. **, p < 0.01 (two-tailed Student’s t test). C, CL2-induced suppression of CREB activity was eliminated by a phosphorylation-deficient TORC2 mutant. An active (∆Dx-CL2ΔC), but not a kinase-dead (∆Dx-CL2KD) CL2 is able to induce a significant reduction in CRE-dependent luc reporter activity in the presence of wild-type (WT) TORC2. However, such kinase-dependent inhibition of CRE-mediated transcription is abolished in the presence of a phosphorylation-deficient TORC2 mutant (TORC2-S171A). Remarkably, a kinase-active form of CaMK2α, CaMK2ΔC, can elicit inhibition of CRE-dependent transcription, irrespective of the TORC2 status, consistent with the idea that CaMK2-dependent inhibition may occur by a mechanism distinct from that mediated by CL2 and TORC2. Each data point on the graphs represents means ± S.E. calculated from triplicates. The results shown are representative of multiple independent sets of experiments. ***, p < 0.001 (one-way ANOVA with Tukey post hoc test).

with assembled microtubules (64–66). The high homology (>70% identity, data not shown) between doublecortin and the doublecortin-like domain (Dx) of either CL1/DCAMKL1 or CL2/DCAMKL2 strongly indicates that these also possess microtubule-associated protein-like activity. In contrast, a Dx-like motif in CLR/DCAMKL3 had only a marginal homology (<40% identity in the most homologous stretch, data not shown), suggesting that CLR may not fully share such activity.

Prior studies of DCAMKL1 have suggested that the Dx-containing DCAMKL1 isoforms are highly expressed in cortical plate neurons as well as migrating neurons (50–52, 64) and down-regulated during development (67), whereas the expression of the shorter variants is noted in adult brain (see for instance, Ref. 28 and this study), although a quantitative comparison for all the splice variants has proven to be difficult (30, 67). In contrast to the case of CL1, all isoforms of CL2/DCAMKL2 possessed a microtubule-interacting Dx. Consistent with a previous report (53), Dx-containing CLick2 was largely co-localized with microtubules in the dendrites and in growing axons, both in hippocampal and cerebellar granule neurons (supplemental Fig. S2, A and B). One possible role for the Dx in CL2 may be to target and restrict a major portion of the kinase activity of CL2 to the microtubule-enriched compartments in the developed neurons, via a direct microtubule-dependent polarized trafficking mechanism or by facilitated targeting through an enrichment of putative targeting molecules. Although our data suggest that it is unlikely that the partial and dysfunctional Dx in CLr may share an intact microtubule-anchoring function, it remains to be seen whether the N terminus of CLr is required for proper cell targeting of CLr in living neurons via other interacting molecules.

One salient feature of the classic CaMKs, such as CaMKI/CaMKIV and CaMKII, i.e. the striking Ca2+/calmodulin dependence of their kinase activity, is largely absent in CL1 and CL2 (26, 48, 53). Consistently, both wild-type CL1 and CL2 demonstrated intact myelin basic protein as well as autophosphorylating activities in the absence of calcium (data not shown). In this respect, it is worth mentioning that one major member of the CaMK family, CaMKKβ, is shown to possess a degree of Ca2+/CaM-independent activity (19, 47). It thus remains to be seen in future studies whether a limited amount of Ca2+/CaM dependence could conversely be reacquired by CL1/CL2/CLR under certain conditions.

Furthermore, another common characteristic previously found for activated CaMKI/CaMKIV and CaMKII, namely their ability to functionally stimulate CREB via phosphorylation of Ser-133 (31–37, 46, 47), was missing in CLRα/CLRβ/CLRβ/CLR (Figs. 5–7 and data not shown). In a previous report, Silverman et al. (26) noted that the rat ortholog for CLRβ, CPG16, is able to diminish CRE-dependent gene expression, but the mechanism underlying this phenomenon was not examined further. The present study clearly demonstrates: 1) that the kinase activity in CL1/CL2/CLR is responsible for suppression of stimulated CREB-dependent transcription, 2) that the kinase effect on transcription is unlikely to be mediated by either inhibition of Ser-133 phosphorylation, stimulation of phospho-Ser-133 dephosphorylation, or phosphorylation of an inhibitory Ser-142 site, and finally 3) that the kinase effect bypasses CREB and is likely mediated by phosphor-Ser-171-TORC2. Previous work suggests that TORC2 phosphorylation may occur in the cytoplasm thus favoring its nuclear exclusion and inhibition of CREB activity (61, 62). Whether this is indeed the case in the neuronal context still remains to be elucidated.

These lines of evidence also highlight the fact that, despite the structural homology found in the catalytic and activation domains between the DCAMKL subfamily and the CaMKI/CaMKIV and CaMKII subfamilies, the functional consequence of kinase activation in intact cells was dramatically opposite at
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the level of CREB regulation. Such a dichotomy could result, in principle, either from a distinct kinase substrate specificity or a completely different mode of kinase regulation (e.g. involvement of a completely distinct regulatory molecule, such as an upstream kinase or an auxiliary subunit), or both. Previous studies using synthetic substrate peptides and in vitro phosphorylation assays have reported mostly overlapping peptide selectivity or substrate recognition motifs between CaMKI/CaMKIV and DCAMKL class of kinases appears to regulate components of the CREB-dependent transcriptional machinery in a manner clearly distinct from that of the CaMKI/CaMKIV- and CaMKII-like classes of CAMK. Further work is needed to fully resolve the similarity and the distinction between the various branches of CAMK family genes in the context of living neurons.

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