Molecular Cloning and Characterization of CLICK-III/CaMKIγ, a Novel Membrane-anchored Neuronal Ca^{2+}/Calmodulin-dependent Protein Kinase (CaMK)∗

Sayaka Takemoto-Kimura‡§, Hisashi Teraï†, Maki Takamoto‡, Shogo Ohmae‡, Shoko Kikumura‡, Eri Segi‡, Yoshihiko Arakawa‡, Tomoyuki Furuyashiki‡, Shuh Narumiya‡, and Haruhiko Bito‡¶**‡‡

From the ‡Department of Pharmacology, Kyoto University Faculty of Medicine, *PRESTO-Japan Science and Technology Corporation, Sakyo-ku, Kyoto 606-8315, and ¶¶Department of Neurochemistry, University of Tokyo Graduate School of Medicine, Bunkyo-ku, Tokyo 113-0033, Japan

During a screen for novel putative Ca^{2+}/calmodulin-dependent protein kinase (CaMK)-like CREB kinases (CLICKs), we have cloned a full-length cDNA for CLICK-III/CaMKIγ, an isoform of the CaMKI family with an extended C-terminal domain ending with CAAX motif (where AA is aliphatic acid). As expected from the similarity of its kinase domain with the other CaMKI isoforms, full activation of CLICK-III/CaMKIγ required both Ca^{2+}/CaM and phosphorylation by CaMKK. We also found that Ca^{2+}/cAMP-response element-binding protein (CREB) was a good substrate for CLICK-III/CaMKIγ, at least in vitro. Interestingly enough, CLICK-III/CaMKIγ transcripts were most abundant in neurons, with the highest levels in limited nuclei such as the central nucleus of the amygdala (CeA) and the ventromedial hypothalamus. Consistent with the presence of the CAAX motif, CLICK-III/CaMKIγ was found to be anchored to various membrane compartments, especially to Golgi and plasma membranes. Both point mutation in the CAAX motif and treatment with compactin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, disrupted such membrane localization, suggesting that membrane localization of CLICK-III/CaMKIγ occurred in a prenylation-dependent way. These findings provide a novel mechanism by which neuronal CaMK activity could be targeted to specific membrane compartments.

Neuronal Ca^{2+} is known to play a critical role as an intracellular second messenger, linking neuronal excitability with many kinds of cellular biological events including synaptic plasticity and neuronal cell survival/apoptosis (1–4). One of the unique features of Ca^{2+} is that its concentration can be dynamically regulated both temporally and spatially (5). Despite a growing knowledge about the critical molecules involved in neuronal Ca^{2+} influx and mobilization (e.g. N-methyl-D-aspartic acid receptors, voltage-gated Ca^{2+} channels, inositol 1,4,5-trisphosphate receptors, and ryanodine receptors), how these are converted to the specific cellular events remains largely unknown.

A significant part of signaling downstream Ca^{2+} is thought to be mediated by calmodulin (CaM), a ubiquitous and evolutionary well conserved intracellular Ca^{2+} receptor (6, 7). Although a large number of molecules have been shown to be targeted and activated by the Ca^{2+}/CaM complex, one subgroup of multifunctional kinases, Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), has been ascribed a prominent role. This is because several unique characteristics of this group of kinases, such as rapid activation by Ca^{2+}, steep Ca^{2+}/CaM dependence, and induction of an autonomous kinase activity following activation, render its members good candidates as molecular devices able to convert a transient burst of synaptic activity into a longer lasting covariant modification of substrate proteins (8–10). Indeed, among the CaMK family members, CaMKIIα and β isoforms, which are present postsynaptically and presynaptically, have been implicated in various kinds of synaptic plasticity and homeostasis (8–11), whereas a CaMKK/CaMKIV cascade has been shown to couple synaptic stimuli with CREB-dependent gene expression (4, 12, 16). The ability of the CaMKI isoforms and a related kinase, CLK, to phosphorylate neuronal substrates, such as synapsin I and CREB in vitro, has been demonstrated so far (13, 17, 18). However, little is yet known about the physiological role of CaMKI, although CaMKIα and CaMKIβ have been shown to be expressed both in neural as well as non-neural peripheral tissues (13, 17–21).

In this study, we report the molecular cloning of mouse full-length CLICK-III/CaMKIγ (occasionally abbreviated to CLICK-III in this paper), an isoform of CaMKI family, that has a longer C-terminal region terminating with a CAAX motif (where AA is aliphatic acid). This motif has been shown to be conjugated with isoprenoid lipids, thereby allowing proper tar-

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‡ Predoctoral fellows from the Japan Society for Promotion of Science.
¶ Postdoctoral fellows from the Japan Society for Promotion of Science.
**‡‡ To whom correspondence should be addressed. Tel.: 81-3-3841-3559; Fax: 81-3-3814-8154; E-mail: hbito@m.u-tokyo.ac.jp.

§ The abbreviations used are: CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CREB, Ca^{2+}/cAMP-response element-binding protein; CLICKs, CaMK-like CREB kinases; VHR, ventromedial hypothalamus; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; RACE, rapid amplification of cDNA ends; HA, hemagglutinin; GFP, green fluorescent protein; EGFP, enhanced GFP; wt, wild type; PBS, phosphate-buffered saline; MBP, myelin basic protein; HBSS, Hank’s balanced salt solution; CNS, central nervous system; CeA, central nucleus of amygdala; CMV, cytomegalovirus.
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geting of various signaling molecules to cellular membrane compartments (22, 23). CLICK-III was identified during a screen for novel putative CaMK-like CREB kinases (CLICKs), as a kinase homologous to CLICK-I and CLICK-II (the cloning and characterization of CLICK-I and -II will be described elsewhere). The kinase activity of CLICK-III was similar in many respects to CaMkIIα and related kinases. Thus, full activation of CLICK-III required both Ca2+ and CaM and phosphorylation by a CaM KK (24–31). In addition, Ca2+/CAMK-response element-binding protein (CREB) (4, 32) was a good substrate for CLICK-III, at least in vitro. Interestingly, CLICK-III transcripts were most abundant in neurons, with the highest levels in limited nuclei such as the central nucleus of the amygdala (CeA) and the ventromedial hypothalamus (VMH). Furthermore, CLICK-III was found to be anchored to membrane compartments, consistent with the presence of the CAAAX motif at its C-terminal end. A point mutation in the CAAAX motif and treatment with compactin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (33), disrupted such membrane localization, suggesting that membrane localization of CLICK-III occurred in a prenylation-dependent way. These findings provide a novel mechanism by which neuronal CaMK activity could be targeted to specific membrane compartments.

**EXPERIMENTAL PROCEDURES**

**Cloning and Plasmid Constructions—**Human hippocampal cDNA was prepared from poly(A)+ RNA (Clontech) using Omniscript reverse transcriptase (Qiagen) and oligo(dT) primers. An hCLICK-III DNA was obtained by nested PCR using two primer pairs (F1, 5'-CCA CTC CCT GCAATA AAG CAT CCT C-3' and R1, 5'-CTG CTT AGT AGTG AGA AGA CGC GCA AAG AA-3'), 250 mM ATP, 100 mM KCl, 1 mM MgCl2, 100 mM NaCl, 10 mM dithiothreitol, and 0.4 units of Taq polymerase. Reaction mixtures were amplified for 25 cycles of 94 °C/2 min, 56 °C/2 min, and 72 °C/2 min, and 1 μl was subcloned into a pCR-Blunt vector (Invitrogen). Mouse hippocampal poly(A)+ RNA was purified using Trizol (Invitrogen) and μM ACS mRNA isolation kit (Milltenyl Biotec), and mCLICK-III was subsequently obtained by the 3'-RACE procedure, using SMART RACE cDNA amplification kit (Clontech) following the manufacturer's instructions. A gene-specific 5'-primer for 3'-RACE was designed based on the cDNA sequence of hCLICK-III and that of rat CaMKI and II sites. To construct pd2EGFPN1-CaMKKactiveMyc, a 1.3-kb cDNA fragment corresponding to the full-length coding region of rat CaMKKα was obtained by PCR using primers (F, 5'-AAC TCGAGT GGC CTA GGC CAC CCA-3' and R, 5'-ATT GGA TCC TAC GAG GAG TCT GCC-3'), subcloned into a p BluescriptII KS(+) vector (Stratagene), and inserted into a p2EGFPN1 vector (Clontech) cut at Xhol and BamHI sites, and thereafter transferred in-frame to pEGFP-C1 vector (Clontech) at EcoRI and BamHI sites. All inserts in the expression vectors were verified by sequencing.

**Northern Blotting—**For Northern blot analysis, a total RNA blot filter was purchased from Seegene (Mouse Brain Aging Blot), and total RNA blot filters were obtained from Clontech (all other blots). Double-stranded probe templates, corresponding to the unique sequence of CLICK-III at the C-terminal region, were generated by PCR using primers (F, 5'-AAG CCT GAG AAA CTT GAC CCA CCA G-3' and R, 5'-TTC ACC AGA CCC AAG CGG CTG CTG-3'), and inserted into a p BluescriptII KS(+) vector for CLICK-III. The probes were labeled with [α-32P]dCTP using Ready-to-Go DNA labeling beads (Amersham Biosciences) and hybridized with the filters in ExpressHyb Hybridization Solution (Clontech) at 68 °C. The filters were washed four times in 0.5% SDS, 2× SSC for 10 min at 50 °C, washed once in 0.1% SDS, 0.1× SSC for 40 min at 50 °C, and subjected to x-ray film autoradiography.

**Hybridization—**Hybridization conditions were optimized from 2-month-old female ICR mice and processed for in situ hybridization as described previously (34). N-terminal (231 bp) and C-terminal (374 bp) fragments were amplified by PCR (5'-GCA GCT TCA ACT CTG GAG G-3' and 5'-TAG GCT GCT GTC CCG GAA GG-3' for N-terminal fragment, 5'-ATG AAC CTG CAC AGC CCC AGT G-3' and 5'-TTA TGG GCC TTT CTG AAG AGG-3' for C-terminal fragment) and subcloned into p BluescriptII KS(+) vector at the SalI site (35). Labeled riboprobes were generated using T7 RNA polymerase (Stratagene) and [α-32P]CTP.

**CaM-Sepharose Binding Assay—**COS-7 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Cells were subcultured in 12-hour dishes 12 h after confluence and incubated at 37 °C in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Cells were collected in modified Eagle medium containing 10% fetal calf serum. Cells were collected in modified Eagle medium containing 10% fetal calf serum, poly(A)+ RNA (Clontech) following the manufacturer's instructions. A gene-specific 5'-primer for 3'-RACE was designed based on the cDNA sequence of hCLICK-III and that of rat CaMKI and II sites. To construct pd2EGFPN1-CaMKKactiveMyc, a 1.3-kb cDNA fragment corresponding to the full-length coding region of rat CaMKKα was obtained by PCR using primers (F, 5'-AAC TCGAGT GGC CTA GGC CAC CCA-3' and R, 5'-ATT GGA TCC TAC GAG GAG TCT GCC-3'), subcloned into a p BluescriptII KS(+) vector (Stratagene), and inserted into a p2EGFPN1 vector (Clontech) cut at Xhol and BamHI sites. All inserts in the expression vectors were verified by sequencing.

**Immunoprecipitate Kinase Assay—**COS-7 cells were plated onto 6-well plates at a density of 2 × 10⁶ per well, and 12 h later were transiently transfected with pcDNA3-HAmCL3wt (0.3 μg) or empty vector (0.3 μg), and pcDNA3-HAmCL3dc (0.5 μg), or empty vector (0.3 μg) and pd2EGFPN1-CaMKKactiveMyc (0.6 μg), or empty vector (0.6 μg) using LipofectAMINE 2000 reagent (Invitrogen). After 24 h, the cells were washed twice with ice-cold PBS(−) and lysed in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl2, 1% Triton X-100, 0.5% deoxycholate, and protease inhibitors (Complete tablet, Roche Applied Science). After the protein concentrations were determined using a DC protein assay kit (Bio-Rad), 250 μg of the lysates were incubated with 15 μl of CaM-Sepharose (Amersham Bioscience) in the lysis buffer for 2 h at 4 °C. Beads were washed for six times in the lysis buffer or Ca2+−free lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and protease inhibitors. Lysates were immunoprecipitated with an anti-HA tag monoclonal antibody (1:2500, 12CA5, Roche Diagnostics).

**Immunoprecipitate Kinase Assay—**COS-7 cells were plated onto 6-well plates at a density of 2 × 10⁶ per well, and 12 h later were transiently transfected with pcDNA3-HAmCL3wt (0.3 μg), pcDNA3-HAmCL3dc (0.5 μg), or empty vector (0.3 μg) and pd2EGFPN1-CaMKKactiveMyc (0.6 μg), or empty vector (0.6 μg) using LipofectAMINE 2000 reagent. For immunoprecipitate kinase assay, cells were washed twice with ice-cold PBS(−) and lysed in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 1 mM diethiothreitol, 25 mM NaF, 10 mM β-glycerophosphate, 0.4% sodium pyrophosphate, and R or C for iPLA2 inhibitors. Lysates were immunoprecipitated with an anti-HA antibody (12CA5) and protein-G-Sepharose (Amersham Biosciences). Immunoprecipitates were washed three times in the lysis buffer and washed twice in kinase buffer containing 50 mM HEPES-NaOH (pH 7.5), 10 mM MgCl2, 1 mM Ca2+, 20 mM β-glycerophosphate, 0.02% Nonidet P-40, 1 mM diethiothreitol, and protease inhibitors. Kinase buffer in the presence of 1 μM CaM, 50 μM ATP, 0.5 μM of γ-[32P]ATP in the kinase buffer using 2.5 μg of CREM (AMP-response element modulator) (Santa Cruz Biotechnology) or 5 μM of MBP (Calbiochem) as substrates for 10 min at 30 °C. For kinase assay without Ca2+ or CaM, 1 μM CaM was omitted, and 1 mM EDTA was substituted for 1 mM Ca2+ in the kinase buffer.

**Luciferase Assay—**COS-7 cells were plated onto 24-well plates at a density of 5 × 10⁴ per well, and 12 h later were transfected with pFR-Luc, pFA-CREB, pRL-CMV (200 ng, 50 ng, 40 ng each; Stratagene), and pIREShsHAmCL3dc (100 ng). 24 h after transfection, luciferase

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activity was measured using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The firefly and the Renilla luciferase activities were both measured using SIRIUS luminescence (Berthold). All results were normalized using Renilla luciferase (pRL-CMV), which was co-transfected with the other reporter genes (pFR-Luc and pFA-CREB).

Culture and Transient Transfection of CA1/CA3 Hippocampal Neurons—Culture of mouse CA1/CA3 hippocampal neurons was carried out as described previously for rat (16). A cDNA transfection was carried out at 7 days in vitro using a modification of the calcium phosphate method.3

BODIPY-TR-ceramide Labeling and Fluorescence Microscopy—For BODIPY-TR-ceramide (Molecular Probes) and green fluorescent protein (GFP) imaging, COS-7 cells were plated on Lab-Tek chambered coverglass (4 well, Nunc) and transfected with expression vectors (0.4 μg/well), pEGFP-rCaMKI, pEGFP-mCL3, pEGFP-mCL3C474S, and pEGFP-CLVM using LipofectAMINE 2000 reagent. After 24 h, the cells were rinsed twice in HBSS/HEPES, incubated for 20 min at room temperature with 2.5 μM BODIPY-TR ceramide/bovine serum albumin in HBSS/HEPES. The cells were washed twice with HBSS/HEPES and replaced with pre-warmed fresh medium, followed by a 1-h incubation.

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in a CO2 incubator. To simultaneously acquire GFP and BODIPY-TR-

toceramide images under live conditions, a Carl Zeiss LSM 510 system

equipped with a Carl Zeiss Axiovert 100TV inverted microscope and a

×63 Plan-Apochromat (NA 1.4, oil) objective (Carl Zeiss) was used. For

all images, stacks of multiple Z-scan sections were obtained, and pro-

jected images were calculated off-line using a projection software on the

LSM 510 system. All pseudocolor representations were assembled using

Photoshop version 5.5 (Adobe). For compactin pretreatment, the normal

medium was replaced with a growth medium containing 40 μM com-

pactin (Wako Pure Chemicals) for 12 h. No apparent phototoxicity was

observed under our experimental conditions.

Subcellular Fractionation—COS-7 cells were grown to 70% conflu-

ency on a 10 cm-dish and transfected with each expression vector (4 μg

per well) using LipofectAMINE 2000 reagent. 48 h after transfection,
the cells were washed twice in ice-cold PBS(−), suspended in 250 μl (per

dish) of homogenizing buffer containing 10 mM HEPES-NaOH (pH 7.5),

10 mM KCl, 2 mM MgCl2, protease inhibitors, disrupted in a Potter-

Elvehjem homogenizer with 40 strokes, and adjusted to 0.25 M sucrose.

To remove unbroken cells and nuclei, the homogenate was centrifuged

at 600 × g for 10 min. The supernatant was separated by centrifugation

at 100,000 × g for 60 min. After collecting the resulting supernatant

cytosolic fraction, S), the membrane pellet was washed once in the

homogenizing buffer containing 0.25 M sucrose and again centrifuged at

100,000 × g for 60 min. For the salt wash experiment, the 600 × g

supernatant was divided into two microtubes and separated by ultra-

centrifugation at 100,000 × g for 60 min. The membrane pellet was

resuspended in homogenization buffer containing 10 mM HEPES-

NaOH (pH 7.5), 10 mM KCl, 2 mM MgCl2, 0.25 M sucrose or high salt

homogenization buffer containing 1 M NaCl, followed by incubation on

ice for 1 h, and ultracentrifuged again at 100,000 × g for 60 min. For all analyses, the membrane pellets were finally extracted with the elution buffer containing 25 mM HEPES-NaOH (pH 7.5), 2% SDS, 150 mM NaCl,

protease inhibitors for 1 h at room temperature and centrifuged for 15

min at 15,000 rpm. This supernatant was collected and designated as

crude membrane fraction (P or P'). The volume of the elution buffer was

adjusted to be equal to the volume of input (identical to the 600 × g

supernatant). For Western blot analyses, one-third volume of 4% Laem-

nuillin buffer was added and boiled for 4 min, and 20 μl of each fractions

were subjected to SDS-PAGE. For compacts treatmen, the medium

was replaced with growth medium containing 40 μM compactin for 16 h.

Western Blot Analysis—After SDS-PAGE, the proteins were trans-

ferred onto a nitrocellulose membrane (Optitran BAS-85, Schleicher &

Schuell), and immunoreactive proteins were detected using ECL-Plus

(Amersham Biosciences) with the following concentration of primary

antibodies: anti-HA tag monoclonal antibody (1:2500, 12CA5; Roche

Applied Science), anti-GFP monoclonal antibody (1:1000, 3E6; Molecu-

lar Probes), and anti-phospho-CREB (Ser-133) polyclonal antibody (1:

1000; Cell Signaling). Horseradish peroxidase-linked anti-mouse or an-

tibodies: anti-HA tag monoclonal antibody (1:2500, 12CA5; Roche

Schuell), and immunoreactive proteins were detected using ECL-Plus

(Toyobo) with the following concentration of primary

antibodies: anti-HA tag monoclonal antibody (1:2500, 12CA5; Roche

Applied Science), anti-GFP monoclonal antibody (1:1000, 3E6; Molecu-

lar Probes), and anti-phospho-CREB (Ser-133) polyclonal antibody (1:

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RESULTS

Molecular Cloning of CLICK-III, a Novel Brain-enriched

CaMK—During the course of studying two novel putative

CaMK-like CREB kinase-I and -II (CLICK-I and -II) cloned by
degenerate PCR strategies,2 BLAST search revealed the pres-

cence of a putative human gene product with high homology to

both CLICKs. We named this novel kinase human CLICK-III.

A putative open reading frame of human CLICK-III had been

deposited as a novel rat Ca2+/calmodulin-dependent protein

kinase-like gene, an assembly of presumed exon sequences

obtained from the draft Human Genome Sequence (Gen-

Bank™ accession number AL023754). We cloned a human

CLICK-III (hCLICK-III) cDNA (GenBank™ accession number

AY212935) by PCR from a human hippocampal cDNA pool,

using primer sequences corresponding to the deposited se-

quence, and we confirmed the authenticity of this gene product.

We next used a 3‘-RACE strategy to obtain a full-length mouse

hippocampal CLICK-III cDNA (GenBank™ accession number

AY212936). Mouse CLICK-III (mCLICK-III) showed a high

degree of amino acid identities with cCLKK, a recently reported

CaMK-like kinase (18), as well as with rat CaMKα (13) and

CaMKβ (19) (Fig. 1, A and B). A partial sequence of a rat

ortholog of CLICK-III was previously reported as rCaMKIy (19)

(asterisk, Fig. 1C).

The open reading frame of CLICK-III cDNAs contained a

N-terminal kinase domain, a conserved threonine at position

178 in the activation loop, and an overlapping autoinhibitory/

Ca2+/CaM-binding domain in its middle portion; these domains

showed high homology across the CaMKI-related kinases (Fig.

1B). The C-terminal end of CLICK-III was unusually extended

in comparison with CCLKK, CaMKα, and CaMKβ, suggesting

a unique role for CLICK-III (Fig. 1C). The very C-terminal end

of hCLICK-III and mCLICK-III were, however, divergent, in-

dicating the existence of multiple C-terminal variants (data not

shown).

As shown in Fig. 2A, Northern blot analyses suggested that

in adult tissues, mCLICK-III was highly expressed in the

brain, although heart, testis, and kidney also showed detecta-

ble amounts of hybridization signals. The mCLICK-III tran-

script first became detected at embryonic day 11 (E11), in

parallel with the onset of the development of the central nerv-

ous system (CNS). Its expression level remained constant from

E11 onward, throughout development and during adulthood

FIG. 3. CLICK-III transcripts are expressed in neurons. Dark-

(A, C, and E) and bright-field (B, D, and F) photomicrographs showed

strong signals in the central nucleus of amygdala (A, CeA) and the

ventromedial hypothalamic nucleus (C, VMH) (also see Fig. 2B, III).

Silver grains were present only on neuronal cell bodies (arrows, B and

D). Moderate signals were detected on the layer V pyramidal neurons in

the cerebral cortex and the CA1 pyramidal neurons in the hippocampus

(E). In the hippocampus, non-principal neurons were also labeled with

silver grains (arrows, F). CA1, CA1 area for Ammon’s horn; hi, the hilus

of the dentate gyrus; Gr, the granule cell layer of the dentate gyrus.

Scale bars, A, C, and E, 0.5 mm; B, D, and F, 50 μm.
Fig. 4. Activation of CLICK-III by Ca\(^{2+}\)/CaM and CaMKK. A, mCLICK-III expressed in COS-7 cells is bound to CaM-Sepharose beads in the presence of Ca\(^{2+}\). Lysates from COS-7 cells transfected with either an empty vector (mock) or a mCLICK-III cDNA (HamCL3wt) were incubated with CaM-Sepharose beads. mCLICK-III remained bound to CaM-Sepharose after washing in the presence of 2 mM Ca\(^{2+}\) (Beads, Ca\(^{2+}\)), whereas most CLICK-III was washed out in 0 mM Ca\(^{2+}\), 2 mM EGTA (Beads, EGTA). WB, Western blot. B, immunoprecipitate kinase assays of CLICK-III. COS-7 cells were transfected with an empty vector (mock), a wild type mCLICK-III cDNA (HamCL3wt), or mCLICK-III C-terminal deletion mutant (HamCL3dC) with (+) or without (−) a cDNA encoding a constitutive active form of rat CaMKK (CaMKK). After immunoprecipitation with an anti-HA antibody, kinase assays were performed in the presence (+) or the absence (−) of Ca\(^{2+}\)/CaM using MBP (5 μg) or CREM (2.5 μg) as substrates. Note that both mCL3wt and mCL3dC require CaMKK activity for their full activation. C-terminal deletion mutant of mCLICK-III showed a clear constitutive kinase activity in the presence of CaMKK, independent of the presence of Ca\(^{2+}\)/CaM, whereas the wild type kinase was only active in the presence of Ca\(^{2+}\)/CaM. C, CREM phosphorylation was also detected in a manner similar to \(^{32}\)P incorporation, using an anti-phospho-CREB (Ser-133) antibody. D, the C-terminal deletion mutant of CLICK-III can activate CREB-dependent transcription in COS-7. Cells were transfected with CLICK-III C-terminal deletion mutant (pIRES-HamCL3dC) and reporter genes coding Gal4-CREB and UAS-luciferase and collected, and lysates were extracted to measure the luciferase activity 24 h later. The bars represent luciferase activities relative to that of mock-transfected lysates (pRES) and are shown as means ± S.E. (n = 3). *** denotes p < 0.001.

Fig. 5. GFP-caMKI\(_{\alpha}\) and GFP-mCL3 show distinct subcellular localization in hippocampal neurons. Mouse CA1/CA3 hippocampal neurons were transfected with GFP, GFP-caMKI\(_{\alpha}\), and GFP-mCL3 for 7 days in vitro, fixed 48 h later, and examined by confocal microscopy. Three representative neurons were shown for each construct (#1–#3). GFP showed diffuse distribution across the cell, whereas GFP-caMKI\(_{\alpha}\) was diffusely expressed mainly in the cytoplasm. In contrast, GFP-mCL3 expression was more localized in specific intracellular compartments (arrowheads) and concentrated at the tips of filopodia-like processes (arrows). Bar, 5 μm.

CLICK-III was a CaMKI family member specifically expressed in neurons, with a remarkable abundance in the CeA and VMH. Such specificity in its expression profile particularly stands out.
Enzymatic Properties of CLICK-III—We next tested whether CLICK-III was indeed a Ca\(^{2+}\)/CaM-dependent protein kinase, as suggested from its domain structure (Fig. 1B). To this end, an HA-tagged CLICK-III was constructed and transfected to COS-7 cells. Crude lysates of transfected COS-7 cells were collected and incubated with CaM-Sepharose beads in the presence of 2 mM Ca\(^{2+}\). The beads were centrifuged and separated from the supernatant and then washed several times in the presence or absence of Ca\(^{2+}\). SDS-PAGE analyses showed that HA-immunoreactive bands were mostly recovered with the beads in the presence of Ca\(^{2+}\), whereas they were largely washed out in 0 Ca\(^{2+}\), 2 EGTA (Fig. 4A). Thus, CLICK-III was clearly able to bind CaM in a Ca\(^{2+}\)-dependent manner.

Ca\(^{2+}\)/CaM-dependent regulation of the protein kinase activity of CLICK-III was investigated using an immunoprecipitate kinase assay, with MBP and CREM as kinase substrates (Fig. 4B). Both Ca\(^{2+}\)/CaM and CaMKK cotransfection were required to reach maximal levels of activation of the wild type CLICK-III (HAmCL3wt). In contrast, a C-terminal deletion mutant in which the entire autoinhibitory/Ca\(^{2+}\)/CaM binding domain was removed (HAmCL3dC) showed a constitutive, Ca\(^{2+}\)-independent kinase activity even in the absence of Ca\(^{2+}\), when active CaMKK was cotransfected (Fig. 4B). CLICK-III activity monitored with \([^{32}\text{P}]\text{ATP}\) incorporation into CREM closely paralleled the amount of phospho-CREB Ser-133 immunoreactivity, suggesting that wild type CLICK-III is able to directly phosphorylate CREB at Ser-133 in an Ca\(^{2+}\)/CaM- and CaMKK-dependent manner, at least in vitro (Fig. 4C). We further confirmed that CLICK-III may work as CREB kinase in vivo by using a Gal4-CREB/UAS-luciferase reporter system. Transfection of a constitutively active CLICK-III (pIRES-HAmCL3dC) significantly augmented CREB-dependent gene expression in COS-7 cells (Fig. 4D). The result was normalized using Renilla luciferase (pRL-CMV), which was co-transfected with the other reporter genes (pFR-Luc and pFA-CREB).

Taken together, these data indicated that the enzymatic properties of CLICK-III recapitulated all major features of CaMKI isoforms, such as dual regulation by Ca\(^{2+}\)/CaM and CaMKK, and activation of CREB pathway in vitro and in a heterologous system.

Membrane Localization of CLICK-III in Hippocampal Neurons and in COS-7 Cells—As we failed to raise high titer antibodies against CLICK-III, we expressed GFP-tagged CLICK-III in hippocampal pyramidal neurons 7 days in vitro, and we monitored its subcellular distribution in fixed (Fig. 5) or live samples (data not shown). Under either condition, GFP alone was diffusely distributed throughout the cytoplasm and the nucleus, whereas GFP-rCaMKI\(\alpha\) remained largely excluded among all CaMKs presently identified (16–21, 35–37).

### Table I

| C-terminal sequences of Ras proteins and mCLICK-III | CAAX motifs are shaded with a gray box. |
|----------------------------------------------------|----------------------------------------|
| mCLICK-III                                         | GSTHCRGGقتVTCLVM                       |
| H-ras                                              | PDESQPGCMSCKCVL3                       |
| N-ras                                              | SDDGTQGCMLPCVVM                        |
| K-ras(4B)                                          | DGKKKKKKSHTKCVIM                       |

Fig. 6. Localization of GFP-mCL3 with the Golgi complex and plasma membranes in COS-7 cells. A, COS-7 cells transfected with GFP, GFP-rCaMKI\(\alpha\), and GFP-mCL3 were stained with a Golgi-specific vital dye (BODIPY TR-ceramide) and examined under live conditions. Projected images from stacks of z-planes (Merge and TR-ceramide) and single z-plane images taken near the bottom, middle, and top of the cells showed localization of CLICK-III to the Golgi complex (arrows) and to the plasma membranes (arrowheads). Green, GFP image; red, BODIPY TR-ceramide. Bar: 10 \(\mu\)m. B, lysates of COS-7 cells transfected with the indicated constructs were fractionated by ultracentrifugation at 100,000 \(\times\) g. The supernatants (S) were collected as cytosolic fractions, and the pellets (P) were washed once and centrifuged again. The resulting pellet was recovered as crude membrane fraction (P). Each fraction was blotted and visualized using an anti-GFP antibody. C, chemiluminescent signal intensities, quantified by a CCD camera-based imaging system, were presented in percentages relative to the total signal intensities detected in both fractions (S + P). The bars represent means \(\pm\) S.E. (\(n = 4\)). ***, \(p < 0.001\).
cluded from the nucleus, in keeping with prior reports (Fig. 5) (15, 16). A striking difference was noted in the subcellular localization of GFP-tagged CLICK-III (GFP-mCL3); GFP-mCL3 distribution was not diffuse but seemed rather associated with intracellular compartments reminiscent of endomembrane systems such as the Golgi complexes or the endoplasmic reticulum (Fig. 5, arrowheads). Furthermore, CLICK-III was also found to be enriched at the tips of thin processes that were most likely filopodia (Fig. 5, arrows); these structures have been proposed to constitute intermediate membrane protrusions that precede the formation of stable dendritic spines during synaptogenesis (38).

To better analyze in detail the potential mechanisms that might underlie the distinct localization of CLICK-III, we overexpressed CLICK-III in COS-7 cells. Ectopic expression of GFP-mCL3 in COS-7 cells confirmed that subcellular distribution of CLICK-III was indeed distinct from that of GFP or GFP-rCaMKIα. Unlike the diffuse distribution of GFP (Fig. 6A, left panels) or the largely cytoplasmic distribution GFP-rCaMKIα (Fig. 6A, middle panels), GFP-mCL3 localization overlapped with the perinuclear Golgi complex, as demonstrated by a high degree of spatial superimposition between GFP fluorescence of GFP-mCL3 and a Golgi-specific vital dye, BODIPY TR-ceramide (Fig. 6A, right panels, arrows). Such a co-localization was not detected between GFP-rCaMKIα, or GFP alone, and the BODIPY TR-ceramide stain (Fig. 6A, upper panels). In addition, a significant amount of GFP-mCL3 signals was seen at the plasma membranes (Fig. 6A, right panels, arrowheads). Consistent with such significant localization of GFP-mCL3 with the Golgi complex and the plasma membranes, a sizable pool of GFP-mCL3 protein was recovered in the membranes fraction (P), after 100,000 g ultracentrifugation (Fig. 6, B and C). In contrast, GFP-rCaMKIα was predominantly present in the supernatants (S) and little in the membrane pellet fraction (P) (Fig. 6, B and C).

A Critical Role for a CAAX Motif in the Localization of CLICK-III to the Golgi Complex and Plasma Membranes—What might be the cause of such distinct subcellular localization between rCaMKIα and CLICK-III? Sequence search identified a putative CAAX membrane-anchoring motif in the very C-terminal end of mCLICK-III (Fig. 1 and Table I). We thus examined whether the CAAX box in mCLICK-III was necessary for its membrane localization. A single amino acid substitution at the putative prenylation site (GFP-mCL3C474S) completely abolished recruitment of mCL3 to the perinuclear Golgi (Fig. 7A, left panels, arrows) and plasma membranes (Fig. 7A, right panels, arrows).
left panels, arrowheads), and this mutant was now diffusely expressed in the cytoplasm (Fig. 7A, middle panels). Conversely, attachment of the CAAX box (CVLM) of the mCL3 to the C terminus of GFP (GFP-CVLM) was sufficient to confer perinuclear Golgi localization to GFP (Fig. 7A, right panels, arrows). We found, however, that GFP-CVLM did not appear to localize well to the plasma membranes, consistent with the notion that residues upstream of the CAAX motif may also play a role in the correct localization and the proper sorting to the plasma membranes (Fig. 7A, right panels) (22, 23). In accordance with these observations, biochemical fractionation experiments showed a significant reduction in the amount of membrane-bound GFP-mCL3C474S especially after high salt wash (P'), whereas GFP-CVLM still remained heavily associated with the membranes (Fig. 7, B and C). Together, these data suggested that the CAAX box of CLICK-III is necessary to localize CLICK-III to the Golgi membranes.

To confirm this point further, mCL3-overexpressing cells were pretreated with compactin, an HMG-CoA reductase inhibitor. As blockade of this rate-limiting step of cholesterol synthesis should also significantly reduce prenylation of the CAAX box cysteine (33), we expected that compactin treatment should mimic the effect of Cys-to-Ser mutation at position 474. Consistently, although vehicle (Me2SO) treatment had no effect on localization of GFP-mCL3, which was strongly detected in association with Golgi and plasma membranes (Fig. 8A, left panels, arrow), an overnight compactin treatment dramatically abolished this membrane anchoring (Fig. 8A, right panels). Prevention of membrane recruitment of GFP-mCL3 and its retention to the soluble fractions by compactin treatment was also verified by using biochemical fractionation as well (Fig. 8, B and C).

Together, these experiments demonstrated that CLICK-III, a neuronally expressed CaMKI isoform, was able to be localized to the Golgi apparatus and plasma membranes, at least in part, via its C-terminal CAAX box in a prenylation-dependent manner.

**DISCUSSION**

CaMK represents a class of the multifunctional protein kinase family, with a particular significance for the physiology of excitable cells such as neurons or smooth muscles (1-10). A better understanding of the role of neuronal CaMKs has been of urgency because CaMK activity has been suggested to be crucial for linking neuronal activity with various types of neuronal plasticity (2-4, 8-11, 39-42). Recent experiments have further revealed that, in fact, various CaMK isoforms play a critical role in establishing distinct types of memory in mammals (9, 12, 43-46). So far, however, most analyses have focused on the role of CaMKIIα, β, and CaMKIV, because these are the most well characterized CaMKs widely expressed in forebrain neurons (35, 36). In contrast, studies concerning CaMKI have lagged behind, in part because the originally isolated CaMKIα has been expressed ubiquitously and little has been shown so far about its physiological role or substrates in neurons (13, 15, 17).

In this study, we have identified a neuronally enriched CaMKI isoform that has the potential to be membrane-anchored. This CaMKI isoform, CLICK-III/CaMKIy, possessed an extended C-terminal domain distinct from known CaMKs. We found that CLICK-III had three particular characteristics that were noteworthy.

First, we confirmed that the activation of CLICK-III required not only Ca2+/CaM but also a phosphorylation by other kinases, presumably by a CaMKK. Such strong CaMKK dependence for its enzymatic activation was qualitatively similar to what was reported previously for CaMKIα (27-29), indicating that the catalytic core of CLICK-III may resemble CaMKIα and may be regulated in a manner identical to CaMKIα. Thus, maximal activation of CLICK-III might only be triggered in close vicinity to an upstream kinase such as CaMKK.

Second, unlike CaMKIα, however, CLICK-III expression was strongly enriched in neurons. More interestingly, examination of its mRNA distribution, by in situ hybridization analyses, revealed a very strong expression in the CeA and the VMH. Such peculiar distribution stands out among all known CaMKs and indicates the possibility that CLICK-III may play a role in the proper function of these nuclei. The CeA has been shown to be a relay for most autonomic outputs and, furthermore, has been associated with the expression of the stimulus-specific state of fear (47). The VMH, on the other hand, has been linked
with control of the homeostasis of feeding and sexual behaviors (48, 49). Future studies are needed to determine whether some of these functions mediated through either Ca²⁺ or VMH might indeed be controlled by the kinase activity of CLICK-III.

Third, our studies have clarified for the first time a mechanism by which CaMKII may be anchored to the Golgi and plasma membranes. A CAAX motif that we identified in the unusually extended C-terminal end of CLICK-III played a critical role in determining the targeting of CLICK-III to the membrane compartments in a heterologous expression system. The high degree of sequence similarity of the CAAX motif of CLICK-III with that of Ras (Table I) and the disruption of membrane anchoring by either a point mutation at the putative prenylation site or by pretreatment with an HMG-CoA reductase inhibitor have suggested that prenylation of the CAAX box may constitute a mechanism through which CLICK-III may be actively sorted to the membranes. The prenyl moiety is derived from the mevalonate/cholesterol biosynthetic pathway. It is interesting to note that this pathway has been shown recently (50, 51) to affect various neuronal phenotypes. Furthermore, it is also notable that many neuronal signaling proteins (e.g., Ras, Rho, parallemmin, and PSD-95) have been modified by the addition of long fatty acid chains including prenylation and palmitoylation (52, 53). These post-translational modifications are suggested to play a critical role for precise membrane localization especially in highly polarized neuronal cells.

What could be the biological significance of such membrane targeting of a CaMKII? In the case of Ras, CAAX box-dependent membrane anchoring was absolutely required to allow proper membrane recruitment of its downstream kinases, c-Raf and B-Raf (23). Similarly, CAAX-mediated localization of CLICK-III to the membranes might represent an advantageous mechanism to help tightly couple upstream signals to local downstream phosphorylation targets. In principle, a direct anchoring of CaMKII activity to various membrane compartments is likely to facilitate the synaptic activity-induced local protein phosphorylation at or very close to the site of Ca²⁺-entry/mobilization. Whether such membrane-delimited excitation-phosphorylation coupling can indeed be triggered by electrical activity of the neurons, either postsynaptically or presynaptically, remains to be studied. Alternatively, or additionally, the presence of a CaMKII in the Golgi apparatus might enable an efficient coupling of synaptic activity with intracellular trafficking of neuronal proteins. Experiments are underway to provide answers to these issues.

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