Molecular Cloning and Characterization of CALP/KChIP4, a Novel EF-hand Protein Interacting with Presenilin 2 and Voltage-gated Potassium Channel Subunit Kv4*

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Alzheimer's disease (AD)1 is a progressive dementing neurodegenerative disorder characterized by a massive deposition of β-amyloid and tau-rich neurofibrillary lesions in the brains (reviewed in Ref. 1 and references therein). A subset of AD is inherited as an autosomal dominant trait, and mutations in three different genes have thus far been linked to early-onset autosomal dominant forms of familial AD (FAD). Among these, presenilin 1 (PS1) and PS2 account for the majority of the early onset FAD (1). PS1 and PS2 genes encode polytopic integral membrane proteins that are predominantly localized in intracellular membranes and span the membrane six to eight times. PS proteins undergo endoproteolysis to give rise to N- and C-terminal fragments, which are the preponderant forms of endogenous PS in vivo (2). These fragments form a heterodimer and are incorporated into high molecular weight (HMW) protein complexes (2–5) that are highly stabilized (t1/2 = ~20 h; Ref. 6), whereas holoproteins of PS are rapidly degraded (t1/2 = ~2 h) (6, 7). The steady-state levels of PS fragments seem to be tightly regulated by competition for shared, but limiting, cellular factors, because overexpression of PS in transfected cells does not increase the overall level of PS fragments and replaces endogenous PS (8).

PS plays an important role in the generation of amyloid β peptides (Aβ) by facilitating intramembranous γ-cleavage of β-amyloid protein precursor (βAPP), as evidenced by the lack of Aβ production and accumulation of βAPP C-terminal stubs in cells established from PS-null mice (9–11). In contrast, FAD-linked mutations in PS increase the production of highly fibrillogenic Aβ42 (12–15), which is the initial and predominantly deposited Aβ species in AD brains (16, 17) and normally consists of only ~10% of total secreted Aβ (18). Moreover, genetic studies in invertebrates and PS-null mice suggested that γ-cleavage-like proteolytic cleavage at site 3 to release Notch intracellular domain (NICD), which is the prerequisite for Notch signaling (reviewed in Ref. 19), also is facilitated by PS. Furthermore, recent findings that the two intramembranous aspartates within the 6th and 7th transmembrane (TM) domains of PS are required for γ-secretase activities (20) and that transition state analogue γ-secretase inhibitors specifically label PS fragments (21–24) strongly support the notion that the PS-containing macromolecular complex catalyzes γ-cleavage and that PS may represent the catalytic subunit of γ-secretase complex. Very recently, ErbB4, a type I single span membrane protein functioning as a tyrosine kinase also was found to be cleaved by γ-secretase (25, 26). These findings suggest that one of the primary functional activities of PS/γ-secretase lies in the intramembranous cleavage of a subset of type I membrane proteins, whereas other functions including regulation of ion fluxes (see Ref. 27 and see “Discussion”) or interaction with cytoplasmic (28) or membrane-bound (29) forms of β-catenin, which apparently do not involve proteolytic activities, also are implicated.
We have shown previously (30, 31) that the C-terminal cytoplasmic region of PS plays an important role in the stabilization and HMW complex formation of PS, which are required for the γ-secretase activity. The mechanistic role of the C terminus of PS in its metabolism and function still remains unknown, but one possibility is that this portion serves as the binding site for the interacting proteins that regulate the metabolism and functions of PS (6, 8). In this paper, we used the yeast two-hybrid system to screen for proteins that interact with the C-terminal region of PS2, and we identified a novel PS-binding protein CALP/KChIP4 (calsenilin-like protein) that belongs to the calsenilin/KChIP family harboring four EF-hand motifs (32). CALP/KChIP4 did not affect the stability or HMW complex formation of PS, nor did it alter γ-cleavage of βAPP or site 3 cleavage of Notch. However, it exhibited a unique character to alter the voltage-gating and inactivation properties of voltage-gated potassium channel subunit Kv4 as observed with other KChIPs.

MATERIALS AND METHODS

**Yeast Two-hybrid cDNA Screening—**MATCHMAKER LexA Two-hybrid System (Clontech, Palo Alto, CA) was used. The manufacturer’s instructions. A cDNA encoding the C-terminal 43 amino acid residues of PS2 (amino acids 406–448) was subcloned into pLexA as a bait. The bait plasmid and the lacZ reporter plasmid, p80-lacZ, were transformed into the budding yeast Saccharomyces cerevisiae strain EGY48, which contains a genomic LEU2 reporter gene. Then human brain cDNA library in pB4AD (CLONTECH) was transformed using the lithium acetate method. Transformants (4.8 × 10^6 clones) were selected on Gal/β-His–Ura/Tryp/Leu plates, and positive clones were chosen after 7–10 days of culture at 30 °C. The colony-λ β-galactosidase filter assay was performed to exclude false positives. The individual library plasmids of positive transformants were recovered from E. coli strain EGY48, which contains a genomic LEU2 reporter gene. Then human brain cDNA library in pB4AD (CLONTECH) was transformed using the lithium acetate method. Transformants (4.8 × 10^6 clones) were selected on Gal/β-His–Ura/Tryp/Leu plates, and positive clones were chosen after 7–10 days of culture at 30 °C. The colony-λ β-galactosidase filter assay was performed to exclude false positives.

**Northern Blot Analysis—**Total RNA was isolated from cultured COS-1 cells, mouse n2A2 (N2a), or human HEK293 cells by the guanidinium thiocyanate/acid phenol procedure (Stratagene) using the following primers: 5′-GAAGTGC-3′ (forward) and 5′-TTAACAACACCACTGG-3′ (reverse). The reaction solution contained the following reagents: 10 mM Tris-HCl (pH 8.8), 2 mM MgSO4, 10 mM KCl, 10 mM (NH4)2SO4, 400 μM dATP, dCTP, dGTP, dTTP, 1 μg of Cap Site cDNA dT (Nippon Gene Co., Ltd., Tokyo) of human brain by nested PCR (‘Cap Site Hunting’). The following sets of primers were used: 5′-GAT-TGACGGTGGCCATCTC-3′ (1RDT) and 5′-CGATCTAACGTCGAC-GAAC (2RDT) as anchor gDNA, and 5′-TTAACCAACACCTGG-3′ (moror-1) and 5′-TGACGGTGGCCATCTC-GACGGTGGCCATCTC-3′ (31-out) as ‘gene-specific’ reverse primers. The reaction solution contained the following reagents: 10 mM Tris-HCl (pH 8.8), 2 mM MgSO4, 10 mM KCl, 10 mM (NH4)2SO4, 400 μM dATP, dCTP, dGTP, dTTP, 1 μg of Cap Site cDNA dT from human brain (Nippon Gene), 1.25 units PfuTurbo polymerase (Stratagene), 0.5 μM each primers (e.g., 1RDT and moror-1 for 1st PCR and 2RDT and 31-out for 2nd “nested” PCR), 5 μl of GC-Melt (CLONTECH). The first PCR was performed for 35 cycles, with each cycle consisting of denaturation for 60 s at 94 °C, annealing for 30 s at 60 °C, and extension for 90 s at 72 °C. The second PCR was performed in the same buffer, using 1 μl of the first PCR products as a template and the same program as the 1st PCR. Specific PCR products were subcloned into pCR-Script Amp SK (+) (Stratagene) and subjected to sequencing. To obtain a mouse CALP cDNA, we performed 5′-RACE or 3′-RACE using mouse brain Marathon-Ready cDNA library (CLONTECH) and CALP-specific primers. The isolated PCR fragments were subcloned and sequenced.

**Construction of Expression Plasmids—**Full-length cDNAs encoding wild-type (wt) or N141I FAD mutant (mt) human PS2 in pC3DNA (Invitrogen) were obtained as described (15). cDNAs encoding full-length human CALP (II-CALP) and NΔ-CALP were generated using PfuTurbo polymerase, and the following oligonucleotides anchored with XhoI (both 5′ and 3′ ends) sites were used as PCR primers: 5′-ggg gag tac gct ega gta tet cg-3′ for fl-CALP, 5′-ccc ggc ggg gaa gaa cag cgg gag gaa cgg gag cgg gag-3′ for ΔN-CALP as a sense primer, and 5′-ggg ccc tgt gaa ata acc aa-3′ as an antisense primer, respectively. The amplified cDNAs were subcloned into pGEX-6P-1 (Amersham Biosciences) and performed a QuikChange™ site-directed mutagenesis according to the QuikChange protocol (Stratagene) using the following primers: 5′-ggc tgg tgg aag aag tgc gag cag cag cgc cac cag cag-3′ for the 2nd EF-hand; 5′-att aat ttt ttt gaa ata nta gat gac tgc tat tgc-3′ for the 3rd EF-hand; 5′-tct cag cgg cta cgg cag ctt ggg acc ata-3′ and 5′-tat gtt gag aac ggc act ttt gtt ggt ctt gtt ctt gtt ctt gtt-3′ for the 4th EF-hand, respectively. To assess the electrophysiological property of CALP, cDNA fragment encoding fl-, ΔN-, or Efl-CALP was amplified by PCR using AmpliTag Gold DNA polymerase (Applied Biosystems) and subcloned into pTracer-CMV2 (Invitrogen). To express the CALP protein in E. coli, cDNA encoding fl- or Efl-CALP fused to glutathione S-transferase (GST) was generated by subcloning the amplified PCR fragments into pGEX-6P-1 (Amersham Biosciences). All constructs were sequenced. cDNAs encoding rat KChIP2s, KChIP2l, KChIP4, human PS2, and CALP/KChIP3 in pcDNA3.1335–35) or mouse NotchΔE in pCS2+ MT vector were described previously (36).

**Immunoprecipitation and Immunoblot Analysis—**To prepare immunoprecipitates, cells were lysed using LipofectAMINE reagent (Invitrogen), N2a cells lines stably expressing wt or mt PS2 were generated as described (15). N2a cells lines stably co-expressing PS2 and CALP were generated by transfecting the cDNAs encoding fl- or Efl-CALP (AGAP II and CALPII) into N2a cells. The anti-rat Kv4.2 rabbit polyclonal antibody was purchased from Roche Diagnostics. Immunoprecipitates were collected, heated to 37 °C for 30 min, and analyzed by immunoblotting.

**Antibodies, Immunoprecipitation, Immunoblot Analysis, and Immunofluorescence Microscopy—**The following rabbit and mouse polyclonal antibodies were used: anti-G2N4 against GST fused to 2–59 of human PS2, anti-G2L, and anti-G3L against GST fused to amino acids 301–361 of human PS2, anti-G1Nrv2 and anti-G1L3 against GST fused to amino acids 391–457 of human PS1, respectively (37), anti-N-CALP against a synthetic peptide corresponding to residues 4–21 of human CALP216 conjugated to keyhole limpet hemocyanin at the C terminus. Anti-rat Kv4.2 rabbit polyclonal antibody was purchased from Chemicon. A mouse anti-c-Myc monoclonal antibody (9E10) was purchased from Roche Diagnostics. For immunoprecipitation, cells were lysed by TSCC (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% CHAPS, Complete protease inhibitor mixture (Roche Diagnostics)) and passed through a 27-gauge needle. The solubilized samples were pre-cleared with protein G-conjugated agarose (Invitrogen) for 1 h at 4 °C, reacted with antibodies overnight, followed by incubation with protein G-conjugated agarose (Invitrogen) for 1 h at 4 °C and washed four times with TSCC (TSCC without CHAPS). Immunoprecipitates were collected, heated to 37 °C for 30 min, and analyzed by immunoblotting. For immunoblot analysis, cells were lysed in 2% SDS sample buffer and brieﬂy sonicated. The samples were separated by SDS-PAGE without previous boiling, transferred to polyvinylidene difluoride membrane, and analyzed by immunoblotting as described (15, 30). For immunofluorescence microscopy, transiently
transfected COS-1 cells were cultured on glass coverslips, fixed, immunostained, and viewed with a confocal laser scanning microscope (FLUOVIEW, Olympus, Tokyo) as described (38, 39), except that secondary antibodies conjugated with Alexa Fluor 488 or 594 (Molecular Probes, Eugene, OR) were used.

**Fractionation of Mouse Tissues and Cells**—Cerebrum, cerebellum, midbrain, heart, lung, liver, kidney, thymus, spleen, testis, and skeletal muscle were dissected from adult mice (7 weeks age) immediately after decapitation, homogenized on ice in 3 volumes of TSI (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 mM diisopropyl fluorophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml antipain, 0.1 μg/ml leupeptin, 1 μg/ml Nα-p-tosyl-L-lysine chloromethyl ketone, 1 mM EDTA) in a Polytron homogenizer (Hitachi, Japan) and centrifuged at 352,000 g for 20 min. The pellets were resuspended in TSI, briefly sonicated, incubated for 30 min on ice, and centrifuged. Each supernatant or pellet was collected, and protein concentration was determined by BCA protein assay (Pierce). Fractionation of protein complexes of different molecular masses was performed by glycerol velocity centrifugation as described previously (31).

**Quantitation of Aβ by Two-site ELISAs**—Two-site ELISAs that specifically detect the C terminus of Aβ were used as described (15, 30, 31, 38–40). BNT77, which was raised against human Aβ-(11–28) and recognizes full-length as well as N-terminally truncated Aβ of human and rodent types (40), was used as a capture antibody. BA27 and BC05 that specifically recognize the C terminus of Aβ40 and Aβ42, respectively, were conjugated with horseradish peroxidase and used as detector antibodies. Culture media were collected after incubation of 24 h and subjected to BNT77/BA27 or BNT77/BC05 ELISAs.

**Electrophysiology**—Whole-cell voltage clamp was applied to single HEK293 cells with patch pipettes using a CEZ-2400 (Nihon Kohden, Tokyo) amplifier as reported previously (33). A type K+ current (IK) was observed in neither native nor GFP-alone transfected HEK293 cells. GFP signals were detected by use of GFP longpass filter (Nikon, Tokyo). All experiments were done at room temperature (23 ± 1°C). Membrane currents were monitored and stored as reported previously (33). Cell capacitance was measured from the integration of capacitive transient

**Table I**

**Interaction assay of CALP with PS C terminus using LexA yeast two-hybrid system**

Amino acid sequences corresponding to various portions of C-terminal domain of PS2 or PS1 used as baits are shown in the 1st column. Full-length CALP was used as a prey, and an empty vector pB42AD was used as a blank (middle column). p53 and SV40-large T antigen were used as a positive control. Results of β-galactosidase (β-Gal) assay (white, negative; blue +++, strongly positive; blue ++, weakly positive) are shown in the last column.

| Bait       | Prey       | β-Gal assay |
|------------|------------|-------------|
| PS2        | pB42AD     | White       |
| 406–448    | CALP       | Blue ++     |
| 406–444    | CALP       | Blue ++++   |
| 406–434    | CALP       | Blue +      |
| 406–431    | CALP       | White       |
| 270–361    | CALP       | White       |
| PS1        | CALP       | Blue ++++   |
| 425–467    | CALP       | Blue ++++   |
| 425–460    | SV40-large T | Blue +    |

**Fig. 1. Amino acid sequence and mRNA expression of CALP.** A, amino acid sequence alignment between KChIP family member proteins including CALP. Residues identical among KChIP proteins are marked by asterisks. Putative four EF-hand motif sequences are shaded. For human CALP, two splice variants with (CALP250) and without (CALP216) an N-terminal insert are shown. B, Northern blot analysis of CALP mRNA in human tissues. Upper panel, the blots were hybridized with a probe generated from human CALP cDNA. Arrowhead indicates CALP mRNA. Lower panel, the blot was probed with radiolabeled β-actin cDNA as a control.
FIG. 2. Expression and protein-protein interaction of CALP and PS2 in cultured cells. A, schematic depiction of CALP and its derivatives used in this study. The names of cDNAs are indicated at the right of each scheme. The CALP \textsubscript{216} polypeptide contains an N-terminal “variable” domain (that is distinct between KChIP members; box), 1st EF-hand domain (that does not bind calcium; lightly shaded box), and the other three EF-hand domains (heavily shaded boxes). Location of immunogen peptide/protein for antibodies used in this study (αCALP2 and αN-CALP) is shown by solid bars below the scheme. Amino acid substitutions at EF-hand domains are shown by an asterisk, respectively. B, \textsuperscript{45}Ca
currents upon small depolarization in each cell. For electrical recordings, HEPES-buffered solution having the following composition was used as the external solution (mM): NaCl, 137; KCl, 5.9; CaCl2, 2.2; MgCl2, 1.2; glucose, 14; HEPES, 10 (pH 7.4). The pipette filling solution contained (mM): KCl, 140; MgCl2, 4; Na2ATP, 5; EGTA, 0.05; HEPES, 10 (pH 7.2).

The voltage dependence of I\alpha activation was measured using the conventional double pulse protocol as mentioned previously (41). The membrane potential was changed from −80 to test potentials for 10 ms to activate I\alpha and then to −40 mV to measure the tail current. The tail current amplitude was normalized with the maximum in each cell and was plotted against the test potentials. The data were fitted with Boltzmann equation, and the voltage required for the half-maximal activation and the slope factor were determined from the fitting. The voltage dependence of I\alpha inactivation was also determined by the double pulse protocol (41). I\alpha was activated and inactivated by depolarization from −80 mV to test potentials for 1 s, and then remaining available channels were activated by the following depolarization to +40 mV. The current normalization and the fitting of data with the Boltzmann equation were performed in a similar manner as those for the activation. The double pulse was applied every 30 s.

Statistics—Pooled data were expressed as mean ± S.E., and statistical significance was examined using the unpaired Student's t or Schef- fe's test for two or multiple groups, respectively. In the figures, * and ** indicate statistical significance at p values of 0.05 and 0.01, respectively.

RESULTS
Cloning of CALP/KChIP4, a Novel Interacting Protein with the C Terminus of Presenilins—To identify proteins that inter-
act with the C terminus of PS, we screened the human brain cDNA library by the yeast two-hybrid system using amino acid residues 406–448 of PS2 as bait and obtained >100 positive clones. We found that ~90% of isolated clones in our screen encoded the same polypeptide. BLAST homology analysis revealed that this clone encoded a novel polypeptide showing some homology to calsenilin, which has previously been cloned as a PS C-terminal binding protein (35), as well as to KChIPs that have been identified as components of native Kv4 channel complex (32). We therefore designated this gene as CALP (calsenilin-like protein)/KChIP4 (Fig. 1A). Multiple sequence alignment and BLAST analysis indicated that CALP had high amino acid identity at its C-terminal region with KChIP2 (79.6%) and calsenilin/KChIP3 (77.6%), whereas its N terminus was very divergent from any KChIPs. CALP cDNA derived from our two-hybrid screening contained an in-frame methionine but lacked an apparent Kozak sequence. To isolate the entire CALP open reading frame, we performed plaque hybridization and 5’-RACE using additional human cDNA libraries, and we cloned an alternatively spliced form of CALP harboring an N-terminal insert sequence. However, we failed to detect an upstream sequence as well as an in-frame termination codon even when we used a Cap site cDNA dT library, which is suitable for the determination of transcription start site (42).

We next cloned a cDNA encoding mouse CALP from a mouse brain cDNA library by the 5’- and 3’-RACE method, and we

![Fig. 3. Protein expression pattern of endogenous CALP in mouse tissues.](Image)
Endogenous mouse CALP protein was detected exclusively in central nervous system-derived Triton X-100-soluble (TSXI sup) and -insoluble (TSXI pel) fractions as a 27-kDa protein (arrowhead), which was recognized both with αCALP2 and αN-CALP. TSI, Tris-HCl in saline containing protease inhibitor mixtures; TSXI, TSI containing 1% Triton X-100.

**Figure 3**
Protein expression pattern of endogenous CALP in mouse tissues. Endogenous mouse CALP protein was detected exclusively in central nervous system-derived Triton X-100-soluble (TSXI sup) and -insoluble (TSXI pel) fractions as a 27-kDa protein (arrowhead), which was recognized both with αCALP2 and αN-CALP. TSI, Tris-HCl in saline containing protease inhibitor mixtures; TSXI, TSI containing 1% Triton X-100.
found an in-frame termination codon located upstream of the first ATG codon, the latter being in a similar position to the first ATG codon in human CALP cDNA. Thus, we concluded that the human cDNA cloned by two-hybrid system encompassed the entire CALP open reading frame encoding a 216-amino acid polypeptide (CALP216); human CALP gene also encoded an alternatively spliced form encoding a 250-amino acid protein (CALP 250) with an N-terminal insert, and all mouse CALP cDNA we cloned harbored this insert corresponding to CALP 250 (Fig. 1A). Northern blot analysis of mRNA derived from human tissues revealed that CALP is predominantly expressed in brain (Fig. 1B). Similar results were obtained in Northern blots of mRNA derived from mouse tissues (data not shown). BLAST search of human genome data from the International Human Genome Project of the National Institutes of Health located the CALP gene on Homo sapiens chromosome 4 working draft sequence segment (GenBank™ accession number NT_006138, Locus ID, 80333).

**PS2 Interacts with the C-terminal EF-hand Domain of CALP Independent of Calcium Binding**—We confirmed the interaction of CALP and PS2 by yeast two-hybrid assay using truncated C-terminal fragments of PS2 as baits (Table I). \( \beta \)-Galactosidase filter assay revealed that C-terminal fragments of PS2 corresponding to residues 406–415 or 406–421 showed very weak or no reaction, suggesting that a minimal subdomain essential for PS2/CALP binding was located between residues 421 and 431 of PS2. Similar analysis in PS1 confirmed that CALP interacts with the C terminus of PS1 at a comparable subdomain (i.e. between residues 425 and 460).

We next characterized CALP and its derivatives expressed in cultured cells. For this purpose, we constructed expression
plasmids encoding full-length (fl) CALP, ΔN-CALP lacking the N terminus (i.e., amino acid residues 1–30), that is variable among KChIP family proteins, and EFmt-CALP with the highly conserved Asp and Gly residues within the EF-hand motifs (i.e., Asp-99 and Gly-104 in the 2nd, Asp-135 and Gly-140 in the 3rd, and Asp-183 and Gly-188 in the 4th EF hand) being replaced by Ala, based on human CALP216 (Fig. 2/H11011). The positions of fl-CALP protein is marked by arrowhead. Note that fl- and ΔN-CALP were co-immunoprecipitated with Kv4.2, whereas EFmt-CALP was not.

To confirm the association of CALP with PS2 in vivo, we doubly transfected COS cells with CALP2 or PS2 loop immunoprecipitated fl-CALP as well as ΔN-CALP or EFmt-CALP, suggesting that this antibody recognizes the conserved C-terminal region. In contrast, ΔN-CALP raised against the CALP-specific N terminus reacted exclusively with CALP.

To examine whether CALP affects the γ-secretase activity of PS complex, we measured the levels of secreted Aβ40 and Aβ42 in conditioned media from N2a cell lines stably expressing PS2 and CALP by Aβ C-terminal specific ELISAs (Fig. 4C). In conditioned media of cells expressing mt PS2, the percentage of Aβ42 as a fraction of total Aβ42 (= Aβ42 + Aβ40/total Aβ42) was elevated to ~80%, whereas %Aβ42 in cells expressing wild-type (wt) PS2 was ~20% (Fig. 4C, see percentages indicated above each column). Overexpression of fl- or EFmt-CALP did not alter the absolute levels of Aβ42 secretion of %Aβ42 in N2a cells co-expressing wt or mt PS2.

To examine the effect of overexpression of CALP on site 3 cleavage, we then transiently transfected a cDNA encoding Notch3E that contains the signal sequence, TM domain, and the intracellular domain of mouse Notch-1 harboring C-terminal Myc epitope tags in N2a cells stably overexpressing PS2 and CALP (Fig. 4D) (36). Consistent with our previous findings (31), production of NICD was impaired in N2a cells stably expressing mt PS2, whereas overexpression of wt PS2 had no significant effect on site 3 cleavage of Notch. The proteolytic release of NICD was not affected by overexpression of fl- or EFmt-CALP in cells expressing wt PS2. Moreover, inhibition of NICD production by FAD-linked mutation in PS2 was not...
affected by overexpression of fl- or EFmt-CALP, suggesting that overexpression of CALP had no significant effect on site 3 cleavage of Notch. Collectively, overexpression of fl- or EFmt-CALP did not affect the \( \text{H9253} \)-secretase activities of PS complex.

Electrophysiological Function of CALP in the Regulation of Kv4 Current

To examine if CALP interacts with Kv4 and function as KChIPs, we first examined the interaction of CALP with Kv4.2 in COS cells transiently co-transfected with these two cDNAs. Full-length or \( \text{H9004} \)N-human CALP were co-immuno-precipitated with rat Kv4.2, whereas EFmt-CALP was not, suggesting that the binding of CALP with Kv4 is taking place through, and is dependent on the integrity of, the EF-hand motifs (Fig. 5).

We next examined the electrophysiological functions of CALP, \( \Delta N \)-CALP, and EFmt-CALP in HEK293 cells expressing Kv4.2 (HEK-4.2). Outward currents were measured from HEK293 cells co-transfected with cDNAs encoding Kv4.2 and GFP, which were detected by the fluorescent signal. Although the cDNA ratio of pcDNA3.1 (Kv4.2) and pTracer-CMV2 (GFP) used for the co-transfection was 1:4, all the HEK cells having GFP signal (\( n > 50 \)) in this study (HEK-4.2/pT) showed substantial \( I_A \), which was not detected in native HEK and those transfected with pTracer alone. In HEK-4.2/pT, typical A-type \( K^+ \) currents (\( I_{A}^\prime \); early inactivating \( K^+ \) current) were activated by depolarization from a holding potential of \(-80 \text{ mV} \) to various potentials in a range of \(-70 \) and \(+40 \text{ mV} \) by \( 10-\text{mV} \) steps. Clamp pulses were applied once every \( 15 \text{ s} \). Current-voltage relationships of current density were obtained from the results typically shown in A. The current density was determined by dividing the peak amplitude of \( I_A \) with cell capacitance. pT, fl-CALP, \( \Delta N \)-CALP, and EFmt-CALP indicate the relationships obtained in Kv4.2+pTracer, Kv4.2+fl-CALP, Kv4.2+\( \Delta N \)-CALP, and Kv4.2+EFmt-CALP, respectively. The number in parentheses indicates the number of cells used in each group.

The time courses of \( I_A \) activation and inactivation in HEK-4.2/pT or \( \Delta N \) appeared to be slower than those in HEK-4.2/pT or EFmt under the peak-matched comparison (not shown). Based on exact analyses, the inactivation phase of \( I_A \) at potentials positive to \(-10 \text{ mV} \) was well fitted by sum of two exponential components in all four groups (Equation 1).

\[
\frac{I(t)}{I_{\text{max}}} = A_f \cdot \exp\left(-\frac{t}{\tau_f}\right) + A_s \cdot \exp\left(-\frac{t}{\tau_s}\right) + A_c \quad (\text{Eq. 1})
\]

where \( \frac{I(t)}{I_{\text{max}}} \) is the relative amplitude of \( I_A \) as the function of \( t \) versus the maximum. \( A_f \), \( A_s \), and \( A_c \) values are the relative contributions of the fast and slow inactivating components at time
Table II
Electrophysiological parameters of $I_A$ and cell capacitance in HEK293 cells expressing Kv4.2 and GFP (Kv4.2), or Kv4.2, GFP, and one of $\beta$-, $\Delta N$-, or EFmt-CALP (Kv4.2+\beta-CALP, Kv4.2+$\Delta N$-CALP, and Kv4.2+EFmt-CALP, respectively)

| Cell capacitance | Current density at +40 mV | Inactivation rate | Activation voltage dependence | Inactivation voltage dependence | Recovery from inactivation |
|------------------|----------------------------|------------------|-------------------------------|-------------------------------|----------------------------|
|                  | $pF$                       | $pA/pF$          | $\tau_i$                      | $A_i$                         | $V_{1/2}$ Slope factor |
| Kv4.2            | 23.7 ± 1.7                 | 131.1 ± 6.1$^{a,b}$ | 29.1 ± 2.5$^{a,b}$             | 264.5 ± 44.3                  | 67.4 ± 0.7 (10) 14.5 ± 1.4$^c$ |
|                  | (10)                       | (10)             | (10)                          | (10)                          | (5)                        |
| Kv4.2+\beta-CALP | 21.2 ± 0.8                 | 265.0 ± 33.7$^c$  | 38.9 ± 2.2$^c$                | 213.1 ± 23.8                  | 92.2 ± 2.1 (10) 13.0 ± 1.1$^c$ |
|                  | (10)                       | (10)             | (10)                          | (10)                          | (5)                        |
| Kv4.2+$\Delta N$-CALP | 26.9 ± 2.1                | 289.6 ± 50.9$^c$  | 40.9 ± 3.1$^c$                | 233.2 ± 47.4                  | 91.8 ± 1.3 (10) 16.2 ± 1.8 |
|                  | (8)                        | (8)              | (8)                           | (8)                           | (5)                        |
| Kv4.2+EFmt-CALP  | 27.4 ± 1.7                 | 165.9 ± 21.5     | 22.5 ± 1.4                    | 262.7 ± 22.4                  | 87.3 ± 0.9 (11) 21.0 ± 2.4 |
|                  | (11)                       | (11)             | (11)                          | (11)                          | (4)                        |

$^a$ Versus Kv4.2+\beta-CALP.
$^b$ Versus Kv4.2+\Delta N-CALP.
$^c$ Versus Kv4.2+EFmt-CALP.

Fig. 7. The time course of recovery from inactivation of $I_A$. A–C, a paired-pulse protocol was applied to determine the time course of recovery from inactivation of $I_A$ at ~80 mV (A, Kv4.2+pTracer; B, Kv4.2+\beta-CALP; C, Kv4.2+EFmt-CALP). Cells were depolarized from ~80 to +40 mV for 1 s twice with a certain interval ($\Delta t$). D, summarized data obtained from results typically shown in A–C. The relative amplitude of $I_A$ was plotted as $IP_1/IP_2$ against $\Delta t$ (ms). $IP_1$ and $IP_2$ are the peak amplitude of $I_A$ activated by $P_1$ and $P_2$, respectively. The recovery time course was best described by single exponential function, and the fitted curves are illustrated. $pT$, $\beta$-CALP, $\Delta N$-CALP, and EFmt-CALP indicate the recovery time courses in Kv4.2+pTracer, Kv4.2+\beta-CALP, Kv4.2+$\Delta N$-CALP, and Kv4.2+EFmt-CALP, respectively. The number in parentheses indicates the number of cells used in each group.

0 to the total inactivation, respectively. $\tau_i$ and $\tau_s$ are the time constants of fast and slow inactivation phases, respectively. $A_c$ is the constant component. The sum of $A_f$, $A_s$, and $A_c$ equals the unity (1.0). The $\tau_i$ at +20 mV was significantly increased by the co-expression with $\beta$- or $\Delta N$-CALP but not affected by EFmt-CALP (Table II). Neither $A_f$ nor $\tau_s$ was significantly affected by the co-expression arrangement.

The influence of co-expression with $\beta$-, $\Delta N$-, or EFmt-CALP on the voltage dependence of $I_A$ activation and inactivation was examined using conventional two pulses protocols (see “Materials and Methods”). The relationships between test voltages and the fraction of activation or inactivation of $I_A$ were well fitted by Boltzmann equation in all four groups (not shown). The summarized results of the voltages required for the half-maximal activation or inactivation ($V_{1/2}$) and the slope factors were listed in Table II. Co-expression with $\beta$- or $\Delta N$-CALP significantly shifted or tended to shift the activation and inactivation of $V_{1/2}$ to negative potentials by several mV but that...
with EFmt-CALP did not (Table II).

One of the most striking effects of fl- and ΔN-CALP was the changes in the time course of IA recovery from inactivation. The recovery time course was studied using a conventional paired-pulse protocol (Fig. 7, A–C). The peak amplitude of IA elicited by the second pulse was normalized with the first one and plotted against the interval (t). The recovery time course of IA was well fitted by a single exponential function, regardless of the co-expression arrangement (Fig. 7D). The time constant in HEK4.2/pET at -80 mV was markedly shortened by the co-expression with fl- and ΔN-CALP but not affected by that with EFmt-CALP (Table II).

**DISCUSSION**

Here we describe a novel EF-hand protein that we identified as a binding protein with the C terminus of PS and designated CALP/KChIP4. CALP showed homology to calsenilin, a member of the recoverin superfamily calcium-binding proteins that has been shown to interact with the C-terminal region of PS (35). It has been reported that calsenilin increases the alternative cleavage of 19-kDa PS2 C-terminal fragment as well as the sensitivity for apoptosis (35, 43), and that calsenilin reversed the enhancement in calcium signaling caused by expression of mutant PS1 in Xenopus oocyte (27). However, the significance of the association of calsenilin and PS in relation to the metabolism and γ-secretase function of PS has not been fully understood. In this study, yeast two-hybrid and co-immunoprecipitation analyses showed that CALP interacts with PS in vivo; however, overexpression of CALP did not affect the metabolism and γ-secretase activities of PS complex. Fractionation analysis of CHAPSO-solubilized membrane fractions suggested that CALP is not a stable component of HMW PS complex that represents the active form of γ-secretase. These results suggest that CALP is possibly a transient binding partner of a PS complex that may represent the immature form of a functional PS complex; however, the precise function of CALP and other KChIPs in PS complex should further be examined by eliminating these proteins from cells, for example by knockout or RNA interference strategies.

Recently, it has been reported that calsenilin is identical to KChIP3, which binds to and modulates the density and the properties of Kv4 current (32). Because of the high homology in EF-hand motif of CALP to other KChIPs, we have analyzed the effect of the co-expression of CALP on Kv4 current density. Like other KChIPs (32, 34), CALP interacted with Kv4.2 polypeptide in vitro and altered the voltage-gating and inactivation properties of Kv4.2. Thus, CALP is a PS-interacting protein as well as a novel KChIP protein, which can be designated as CALP/KChIP4. Although the molecular mechanism underlying the modulation of Kv4 currents by KChIPs remains unclear, it has been envisioned that KChIPs bind to the Kv4 N-terminal domain, facilitate trafficking to the plasma membrane, regulate Kv channel turnover, and/or alter the intrinsic channel property (32, 44–46). The increase in Kv4 channel density and the modulation of the channel activities by CALP in central nervous system neurons, where these components are highly expressed in combination, may be critical to characterize the neuronal excitability. These results also raise the possibility that CALP and other KChIP proteins including calsenilin interact with various polytopic membrane protein complexes to regulate their metabolism, trafficking, and/or functions in membranous compartments.

CALP polypeptide carries four EF-hand domains and binds calcium. Some PS-associated proteins (e.g. calpain, calmyrin, and sorcin) also bind calcium, and PS has been implicated in intracellular calcium homeostasis (27, 47–51). In this regard, CALP might have a function in PS-mediated calcium modulation.

Our data indicate that EF-hand domains of CALP, as well as those of other KChIPs (32), are essential in facilitating the functional expression of Kv4, binding to Kv4, and also modulating the channel kinetics and, thereby, may act as a calcium sensor in the regulation of channel activity. Although it has yet to be determined whether calcium is involved in the regulation of PS function in a similar context to Kv4, it will be important to characterize the effect of CALP on capacitative calcium entry, a novel refilling mechanism for depleted intracellular calcium stores and regulated by PS proteins as recently reported (52, 53).

The reason why overexpression of CALP affects the Kv4 current density but not the metabolism and function of PS has yet to be elucidated; a straightforward interpretation of these data would be that CALP is neither the limiting cellular factor that regulates the levels of PS complex nor a component of PS complex that modulates the function of γ-secretase. In this regard, it would be important to see if CALP, Kv4.2, and PS2 form a ternary complex or if Kv4 or PS2 are incorporated into separate complexes with CALP, although our preliminary immunoprecipitation/Western analyses have so far not shown the presence of a ternary complex (data not shown).

In summary, we identified and characterized CALP/KChIP4 as a novel PS- and Kv4-binding protein that belongs to calsenilin/KChIP protein family and modulates Kv4 functions. Further investigations into the molecular mechanism whereby CALP regulates the function of PS and Kv channels will facilitate our understanding of Alzheimer’s disease and normal brain function.

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Molecular Characterization of CALP/KChIP4

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