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Identification and Characterization of CaMKP-N, Nuclear Calmodulin-Dependent Protein Kinase Phosphatase[1]

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Calmodulin-dependent protein kinase phosphatase (CaMKP) dephosphorylates and concomitantly deactivates multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), such as CaMKI, CaMKII, and CaMKIV. In the present study, a nuclear CaMKP-related protein, CaMKP-N, was identified. This protein consisted of 757 amino acid residues with a calculated molecular weight of 84,176. Recombinant CaMKP-N dephosphorylated CaMKIV. The activity of CaMKP-N requires Mn^{2+} ions and is stimulated by polycations. Transiently expressed CaMKP-N in COS-7 cells was localized in the nucleus. This finding together with previous reports regarding localization of CaMKs indicates that CaMKP-N dephosphorylates CaMKIV and nuclear CaMKII, whereas CaMKP dephosphorylates CaMKI and cytosolic CaMKII.

Key words: CaM-kinase, cDNA sequence, nuclear protein, protein kinase phosphatase, protein phosphatase.

Calmodulin-dependent protein kinases (CaMKs) are involved in various cellular reactions by a calcium stimulus (*1–3*). CaMKI (*4, 5*) and CaMKIV (*6–8*) are activated by the phosphorylation of Thr^{177} and Thr^{196}, respectively, in their activation loops by CaMK kinase, and CaMKII (*9–12*) is activated by autophosphorylation of Thr^{286} within the autoinhibitory domain. Recently, we identified a protein phosphatase, CaMK phosphatase (CaMKP), which specifically dephosphorylated these phosphorylation sites of CaMKs (*13–15*). CaMKP is a calyculin A-insensitive, Mn^{2+}-dependent, and poly-L-lysine [poly(Lys)]-stimulated protein phosphatase. Rat CaMKP consists of 450 amino acid residues with a molecular weight of 49,165 and shows slight similarity (19.6%) to PP2Cα (*15*). CaMKP (*15, 16*) and CaMKI (*17*) are localized in the cytosol, but CaMKIV is localized in the nucleus (*18, 19*). The subcellular localization of these proteins suggests the existence of another protein phosphatase related to CaMKP, which should be localized in the nucleus. In the present study, we searched the database with the sequence of CaMKP and found CaMKP-N, a protein which showed similarities to CaMKP. Recombinant CaMKP-N was expressed and characterized.

MATERIALS AND METHODS

Materials—ATP, poly-L-lysine [poly(Lys), average molecular weight 87,000 and 9,600], poly-L-arginine [poly(Arg), average molecular weight 40,000], poly-L-(glutamic acid, lysine,tyrosine) 6:3:1 [poly(Glu,Lys,Tyr) 6:3:1, average molecular weight 23,000], mixed histones (type II-A), myelin basic protein (MBP), and protamine were purchased from Sigma (St. Louis, MO). Calmodulin was prepared as described previously (*20*). [α-^{32}P]dCTP (3,000 Ci/mmol) and [γ-^{32}P]ATP (5,000 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin) were from the Peptide Institute (Osaka). Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto,), Toyobo (Osaka), or New England Biolabs (Beverly, MA). Dulbecco's modified Eagle's medium and fetal bovine serum were from Gibco BRL/Life Technologies (Rockville, MD). Other reagents were of the highest grade commercially available. Human cDNA clones, KIAA1072 (CaMKP-N) and KIAA0015 (CaMKP), were obtained from Kazusa DNA Research Institute, Chiba.

Protein Preparations—Recombinant mouse p42 mitogen-activated protein kinase (MAP kinase, Erk2)-GST (inactive) and recombinant human MEK1 (active) were purchased from Upstate Biotechnology (Waltham, MA). Myosin light chain (MLC) was prepared from chicken gizzard myosin by the method of Perrie and Perry (*21*). Chicken gizzard myosin was prepared by the method of Ebashi (*22*). Protein kinase C (PKC) was purified from rat cerebral cortex according to Woodgett and Hunter (*23*). Recombinant rat CaMK kinase α expressed in *Escherichia coli* (*24*) was purified as described previously (*25*). Recombinant rat CaMKIV mutants, CaMKIV(K71R) and CaMKIV(K71R/

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Abbreviations: CaMK, calmodulin-dependent protein kinase; CaMKP, CaMK phosphatase; DTT, dithiothreitol; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; MLC, myosin light chain; PBS, phosphate-buffered saline; PKC, protein kinase C; poly(Lys), poly-L-lysine; poly(Arg), poly-L-arginine; poly(Glu,Lys,Tyr); poly-L-(glutamic acid,lysine,tyrosine); PP, protein phosphatase.

T196S) expressed in *E. coli* was prepared and purified (*26*).

Preparation of Protein Phosphatase Substrates—A phosphopeptide, pp10 (YGGMHRQETpVDC), containing the sequence around the autophosphorylation site of CaMKII (281–289) was kindly provided by Dr. N. Yumoto [National Institute of Advanced Industrial Science and Technology (AIST), Osaka]. Recombinant rat CaMKIV(K71R) (127 μg/ml) and CaMKIV(K71R/T196S) (187 μg/ml) were phosphorylated by CaMK kinase α (0.24 μg/ml) at 30°C for 20 min in a reaction mixture comprising 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 5 μM calmodulin, 0.4 mM $CaCl_2$, 2 mM dithiothreitol (DTT), and 50 μM [γ-^{32}P]ATP. MLC (200 μg/ml) was phosphorylated by PKC (1.03 μg/ml) at 30°C for 20 min in a reaction mixture consisting of 30 mM Tris-HCl (pH 7.5), 30 mM KCl, 1 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP, 2.02 μg/ml 1,2-dioleoyl-rac-glycerol, 150 μg/ml phosphatidylserine, and 0.5 mM $CaCl_2$ (*27*). MAP kinase-GST (inactive, 20 μg/ml) was phosphorylated by active MEK1 (78 ng/ml) at 30°C for 20 min in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM EGTA, 2 mM DTT, and 50 μM [γ-^{32}P]ATP. The reactions were terminated by adding excess EDTA (12.3 mM) and bovine serum albumin (1 mg/ml), and the phosphoproteins were desalted with a Sephadex G-50 spin column as described previously (*13*) except that the equilibration buffer used consisted of 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 0.1 mM EGTA, and 0.01% Tween 20. The concentrations of the phosphoprotein substrates presented in the text represent the concentrations of ^{32}P bound to the substrate proteins.

Protein Phosphatase Assay—Dephosphorylation of a phosphopeptide substrate was carried out at 30°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM $MnCl_2$, 0.1 mM EDTA, 0.01% Tween 20, 10 μg/ml poly(Lys), and 40 μM of a phosphopeptide substrate. The reaction was started by the addition of CaMKP-N and stopped by the addition of malachite green solution prepared by the method of Baykov *et al.* (*28*). The release of Pi was determined by the malachite green method of Baykov *et al.* (*28*).

Dephosphorylation of a phosphoprotein substrate was carried out at 30°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM $MnCl_2$, 0.1 mM EDTA, 0.01% Tween 20, 10 μg/ml poly(Lys), and 7.35 nM phosphoprotein substrate. The reaction was started by the addition of CaMKP-N and stopped by the addition of SDS sample buffer. The mixture was analyzed by SDS–polyacrylamide gel electrophoresis, followed by autoradiography.

Northern Blot Analysis—Probes were made from cDNA fragments of human CaMKP-N (nucleotides 931–2422, GenBank/EMBL/DDBJ accession number AB028995) and CaMKP (nucleotides 1–2519, D13640) by the random primer method. A human MTN membrane (Clontech, Palo Alto, CA), on which approximately 1 μg each of poly(A)$^+$ RNA isolated from various human tissues had been blotted, was hybridized with the ^{32}P-labeled probes in ExpressHyb hybridization solution (Clontech) at 68°C for 2 h. The membrane filter was then washed with a solution consisting of 0.1× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS at 65°C.

Expression of CaMKP-N in E. coli—*Xho*I and *Nco*I restriction sites were created at the translational initiation site of CaMKP-N cDNA using a mutagenic sense oligonucleotide, 5′-GGTCTGCCCTGGGGCTCGAGCCGCCATGG-CCGGCTGCATC-3′, by the method of Kunkel (*29*). The 2.3-kb *Xho*I–*Nhe*I fragment containing the entire coding sequence of CaMKP-N was subcloned into pUC119 to generate pUC-CaMKP-N.

The 2.3-kb *Xho*I–*Sal*I fragment of pUC-CaMKP-N was inserted into the *Sal*I site of pET28a (Novagen, Madison, WI) to generate plasmid pET-His6-T7-CaMKP-N, which encodes full-length CaMKP-N with N-terminal His- and T7-tags. This construct was introduced into *E. coli* strain BL21-CodonPlus(DE3)-RIL or BL21-CodonPlus(DE3)-RP (Stratagene, La Jolla, CA). The transformed bacteria were grown at 37°C to an A_{600} of 0.5, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.25 mM. After 2.5 h at 30°C, the bacteria were harvested by centrifugation and suspended in SDS sample buffer.

Expression of CaMKP-N in Sf9 Cells—A cDNA fragment that encodes His- and T7-tagged CaMKP-N was excised from pET-His6-T7-CaMKP-N and introduced into a baculovirus, AcNPV, using a Bac-To-Bac Baculovirus Expression System (Gibco BRL/Life Technologies). Sf9 cells infected with the recombinant baculovirus were grown at 27°C in Grace's medium comprising 10% fetal bovine serum, 0.35 g/liter $NaHCO_3$, 100 μg/ml kanamycin, and 2.6 g/liter Bacto tryptose phosphate broth (Difco) (pH 6.1). After 2 to 3 days, the cells were harvested and suspended in Tris-buffered saline [10 mM Tris-HCl (pH 7.5), 150 mM NaCl] containing 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of microbial protease inhibitors (leupeptin, pepstatin A, antipain, and chymostatin), and then disrupted by sonic oscillation. The residue was removed by centrifugation to generate the crude extract. The recombinant protein was purified using Ni-NTA chromatography (Qiagen) according to the manufacturer's instructions.

Expression of Proteins in COS-7 Cells—A cDNA fragment that encodes His- and T7-tagged CaMKP-N was excised from pET-His6-T7-CaMKP-N and inserted into a mammalian expression vector, pEGFP-N1 (CLONTECH), to generate pHis6-T7-CaMKP-N. For expression of GFP-fusion proteins, GFP-CaMKP-N, a 2.3-kb *Xho*I–*Sal*I fragment of pUC-CaMKP-N was inserted into the *Sal*I site of pEGFP-C1 (CLONTECH), to generate pEGFP-CaMKP-N. For expression of a C-terminal deletion mutant, GFP-CaMKP-N(1-573), pEGFP-CaMKP-N was digested with *Bgl*II and *Bam*HI, and the fragment containing the N-terminal half of CaMKP-N (residues 1–573) was self-ligated to generate pEGFP-CaMKP-N(1–573).

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a humidified incubator at 37°C under an atmosphere of 5% CO_2. Transfection was carried out using Trans-IT-LT1 (PanVera Corp., Madison, WI), according to the manufacturer's instructions. In brief, COS-7 cells were plated at 1× 10^5 cells/ml on a 60-mm dish in 3 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After 20–24 h of culture, the cells were incubated for 2–4 h in 3 ml of OPTI-MEM I Medium (Gibco BRL/Life Technologies) containing 6 ml of Trans-IT-LT1 and 3 μg of plasmid DNAs for transfection. After incubation, the cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Analysis was performed 1–2 d after transfection.

Immunocytochemical Analysis—Transfected COS-7 cells

on coverslips were rinsed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde for 30 min, washed three times in PBS, and then permeabilized with methanol for 5 min. After washing in PBS, the cells were incubated with 0.5% skim milk in PBS for 30 min, rinsed with PBS, incubated with 0.5 μg/ml monoclonal antibody against the T7-tag (Novagen) for 16 h, washed three times in PBS, then incubated with 2 μg/ml of fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in PBS containing 0.5% skim milk for 1 h. After washing three times in PBS, the cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA), and examined by fluorescence microscopy under a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY) equipped with a standard FITC filter set, #487909 (Carl Zeiss), using a Zeiss 40× Plan-NEOFLUOR lens (Carl Zeiss). The GFP-fusion proteins were detected either in fixed cells or in living cells using the standard FITC filter set.

Western Blot Analysis—Samples were subjected to SDS–polyacrylamide gel electrophoresis on a 7.5% acrylamide gel, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was blocked with 5% skim milk in PBS for 0.5 h at 24°C, then incubated with 0.5 μg/ml monoclonal antibody against the T7-tag (Novagen) for 1 h, washed several times in PBS, and incubated with 60 μg/ml goat anti-mouse immunoglobulins (IgA+IgG+IgM) conjugated with horseradish peroxidase (Organon Teknika, Durham, NC) in PBS containing 5% skim milk. After washing of the membrane in PBS, the positive bands were detected with the SuperSignal BLAZE chemiluminescent substrate (Pierce, Rockford, IL).

RESULTS

Identification of CaMKP-N—Subcellular localization of CaMKs and CaMKP suggested the existence of another protein phosphatase similar to CaMKP. A similarity search of the DDBJ, EMBL, and GenBank databases using the BLAST program was conducted with human CaMKP as a query. The search revealed that a human cDNA clone, KIAA1072 (accession number AB028995) (*30*) showed strong similarity to CaMKP. This protein, which we named CaMKP-N (N because it existed in the nucleus as described later), consists of 757 amino acid residues with the calculated molecular weight of 84,176 (Figs. 1 and 2). CaMKP-N contains a phosphatase 2C motif (PROSITE no. PS01032) and the phosphatase domain is located in the center of its sequence. In the amino-terminal region, some characteris-

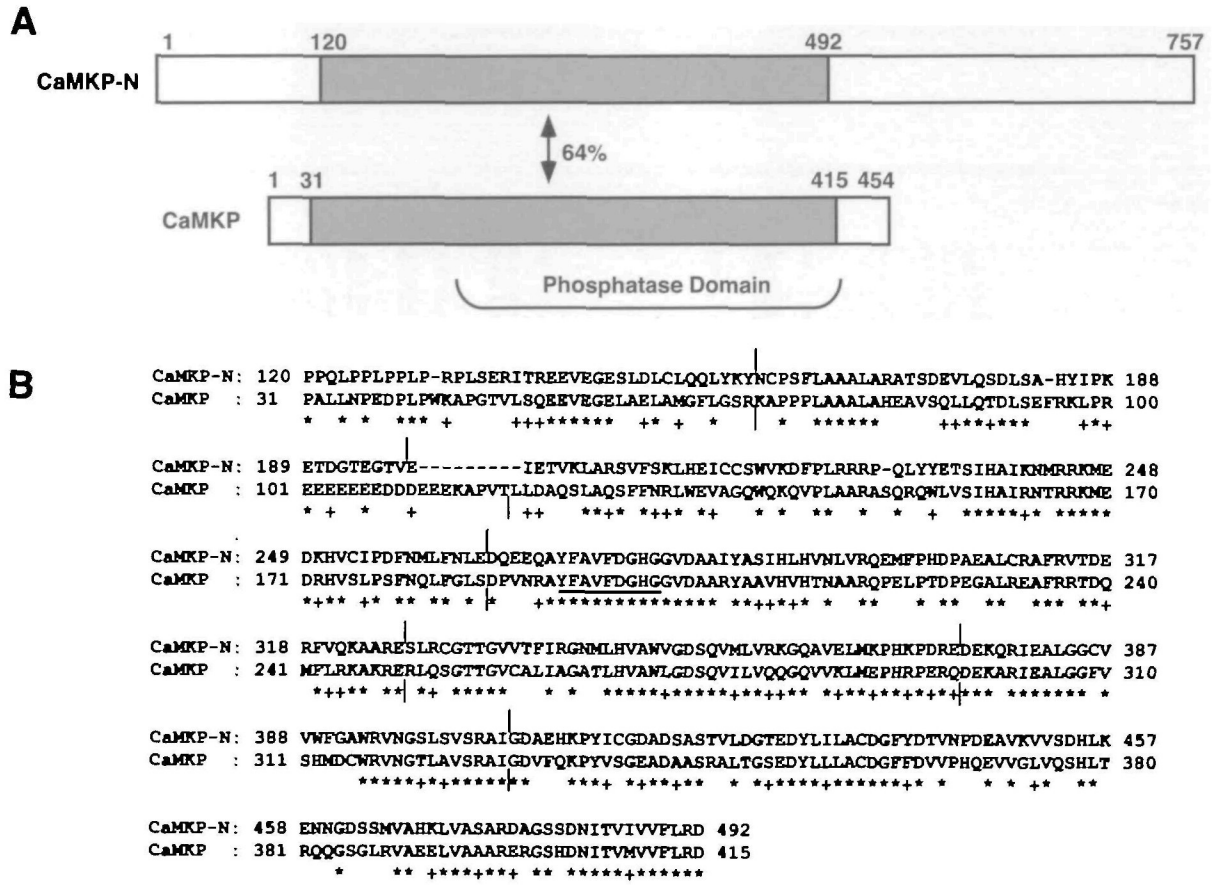

Fig. 1. **Comparison of the amino acid sequence of human CaMKP-N with that of CaMKP.** Schematic representation of CaMKP-N and CaMKP (A) and alignment of the amino acid sequences of CaMKP-N and CaMKP (B) are shown. Only the region that shows significant similarity is aligned. Identical residues are represented by asterisks. Conserved residues are represented by plus signs. Gaps are represented by dashes. The solid underline indicates a protein phosphatase 2C motif (PROSITE no. PS01032). Vertical lines indicate the exon-intron boundaries deduced from the human genome draft sequence.

tic sequences are found. These sequences are repeats of glutamate and proline, a cluster of glutamate residues, and a cluster of proline residues. The similarity (64%) between CaMKP-N and CaMKP was shown in the phosphatase domain and an adjacent N-terminal region (Fig. 1). Unlike CaMKP, CaMKP-N has large extra sequences at the N- and C-termini. CaMKP-N and CaMKP contain the motif of the PPM family (Mg^{2+}- or Mn^{2+}-dependent PP2C-like pro-

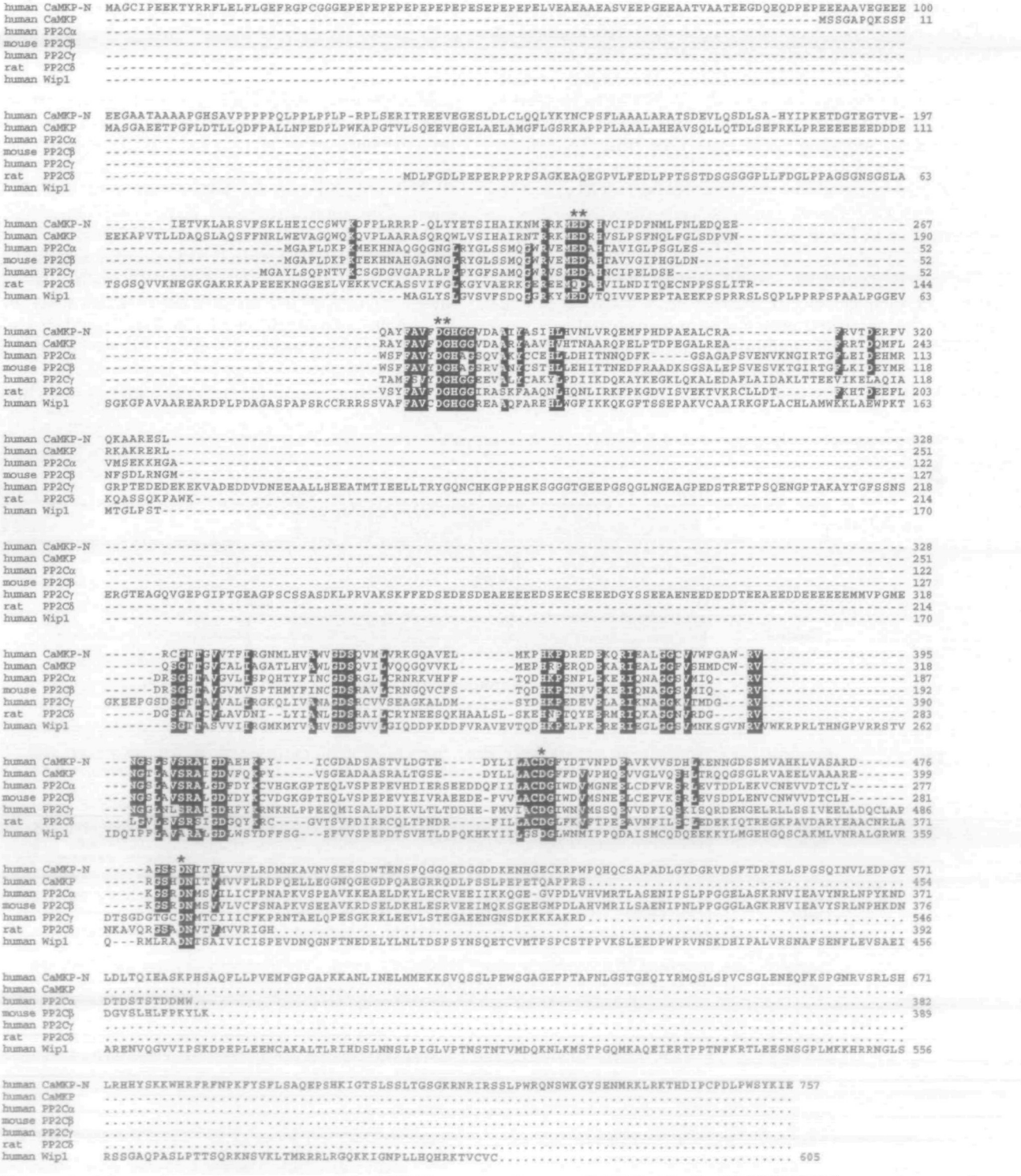

Fig. 2. **Alignment of the amino acid sequences of the PPM family.** The amino acid sequences of the PPM family are aligned. Identical residues in at least five proteins are shown by white letters on a black background. PP2Cγ has a large insertion. The sequences used are human CaMKP (GenBank/EMBL/DDBJ accession number D13640), human CaMKP-N (AB028995), rat PP2Cδ (AF095927), human PP2Cα (S87759), mouse PP2Cβ (D17412), human PP2Cγ (Y13936), and human Wip1 (U78305).

tein phosphatase), indicating these proteins belong to this family of protein phosphatase (Figs. 2 and 3). However, comparison of CaMKP-N with PPM members other than CaMKP shows only weak similarities (less than 20%).

Tissue Distribution of CaMKP-N—Tissue distribution of CaMKP-N was examined by Northern blot analysis of various human RNAs (Fig. 4). A single transcript of 6.5 kb was detected in brain and, after long exposure, also faintly in heart, kidney, and liver (data not shown). Whereas CaMKP-N was mainly expressed in the brain, CaMKP was expressed in all tissues examined, with the highest levels in peripheral blood leukocyte and placenta.

Phosphatase Activity of CaMKP-N—To characterize the enzymatic properties of CaMKP-N, recombinant CaMKP-N was expressed in *E. coli*, Sf9 cells, or a mammalian culture

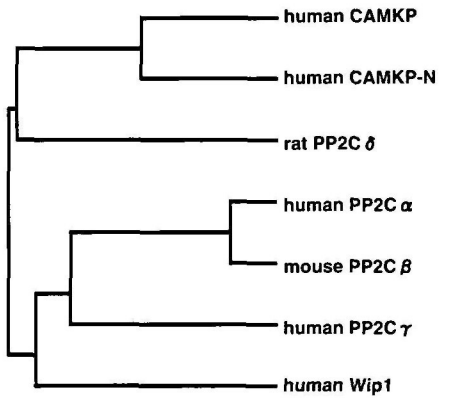

Fig. 3. **Structural relationship between members of the PPM family.** The phylogenetic tree was constructed by use of the Genetyx software (Software Development, Tokyo). The sequences used are human CaMKP (GenBank/EMBL/DDBJ accession number D13640), human CaMKP-N (AB028995), rat PP2Cδ (AF095927), human PP2Cα (S87759), mouse PP2Cβ (D17412), human PP2Cγ (Y13936), and human Wip1 (U78305).

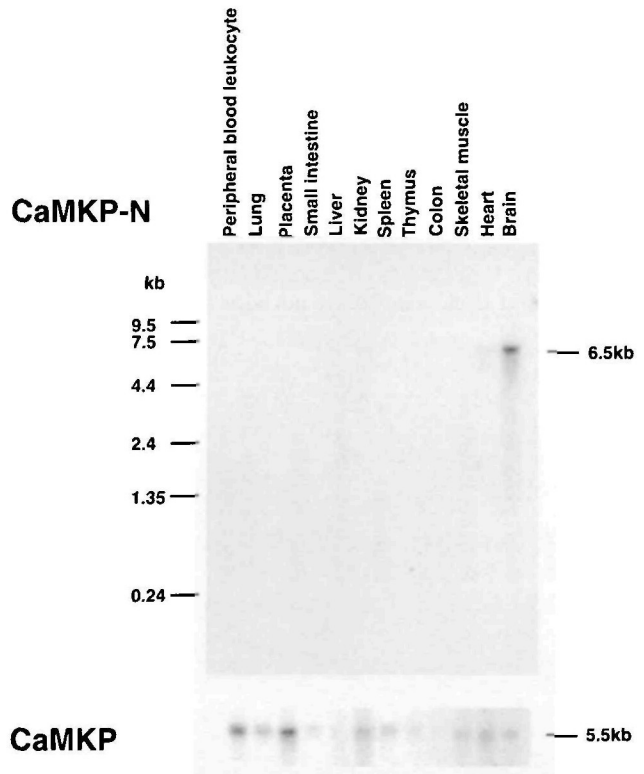

Fig. 4. **Tissue distribution of CaMKP-N and CaMKP mRNAs.** Human MTN membrane (Clontech), on which approximately 1 μg of poly(A)$^+$ RNA isolated from peripheral blood leukocyte (lane 1), lung (lane 2), placenta (lane 3), small intestine (lane 4), liver (lane 5), kidney (lane 6), spleen (lane 7), thymus (lane 8), colon (lane 9), skeletal muscle (lane 10), heart (lane 11), and brain (lane 12) had been blotted, was hybridized with a ^{32}P-labeled probe specific for CaMKP-N (nucleotides 931–2422) or CaMKP (nucleotides 1–2519), and analyzed as described under "MATERIALS AND METHODS." RNA sizes in kilobases are indicated on the left.

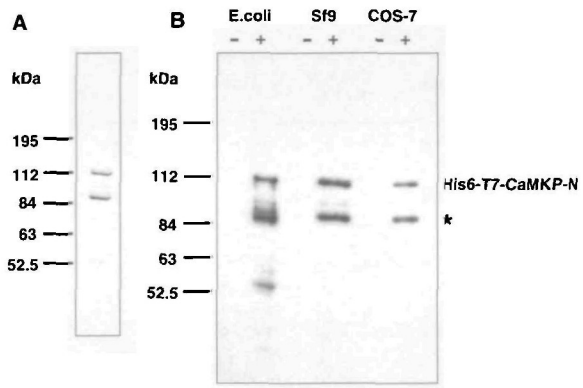

Fig. 5. **Expression of CaM-kinase phosphatase cDNA in *E. coli*, Sf9 cells and COS-7 cells.** (A) Approximately 0.1 μg of CaMKP-N purified from Sf9 cells using an Ni-NTA column was subjected to SDS–polyacrylamide gel electrophoresis on 7.5% acrylamide gel, and stained with Coomassie Brilliant Blue R-250. Molecular masses in kilodaltons are given on the left. (B) Approximately 5 μg of the crude extract from *E. coli*, Sf9 cells and COS-7 cells transfected with His6-T7-CaMKP-N cDNA (+) or nontransfected cells (–) were subjected to Western blot analysis using a T7-tag antibody, as described under "MATERIALS AND METHODS." Molecular masses in kilodaltons are given on the left. A smaller probably proteolytic product, is indicated by the asterisk.

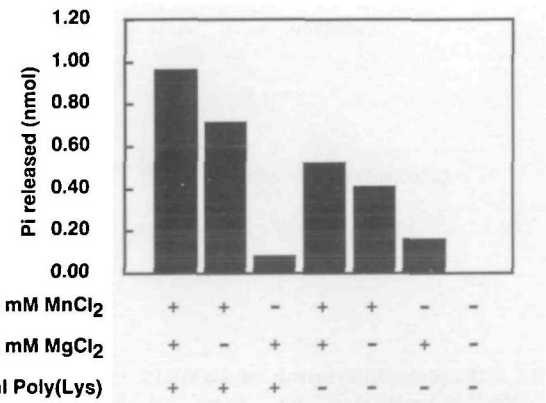

Fig. 6. **Phosphatase activity of CaMKP-N.** Recombinant CaMKP-N (0.2 μg/ml), which had been partially purified from Sf9 cells, was incubated with a phosphopeptide substrate, pp10, in a reaction mixture at 30°C as described under "MATERIALS AND METHODS," with the indicated additions and omissions. After incubation for 30 min, the reaction was terminated, and the phosphatase activity was determined from the released P_i as described.

cell-line, COS-7 cells. His- and T7-tagged CaMKP-N of approximately 110 kDa was detected, as well as a smaller protein, probably a proteolytic product (Fig. 5B). CaMKP-N expressed in Sf9 cells was partially purified using Ni-NTA chromatography (Fig. 5A) and used for further analysis. The sequence homology of CaMKP-N to CaMKP and the conserved PP motif, which is characteristic of the PPM family, indicated that CaMKP-N has phosphatase activity. Since we previously showed that CaMKP requires Mn^{2+} and is activated by polycations (*13, 15*), in this study we examined whether CaMKP-N also exhibits these properties. Using a synthetic phosphopeptide, which consists of the sequence of the phosphorylation site of CaMKII, CaMKP-N was analyzed and was found to show phosphatase activity (Fig. 6). Like CaMKP, CaMKP-N exhibited a preferential requirement for Mn^{2+} ions, and CaMKP-N was activated by poly(Lys).

We then examined the substrate specificity of CaMKP-N and found that it dephosphorylated CaMKIV but not myosin light chains (Fig. 7). Poly(Lys) enhanced the phosphatase activity towards CaMKIV in a similar way to that in the assay using a synthetic peptide. The dephosphorylation site of CaMKIV, Thr^{196}, is located at the activation loop (*25, 31*). We carried out an assay with another protein kinase which is phosphorylated at the activation loop. MAP kinase (Erk2) is phosphorylated by activated MAP kinase kinase (MEK) at Thr^{183} and Tyr^{185} within its activation loop, leading to activation of the kinase (*32*). CaMKP-N dephosphorylated CaMKIV, but not MAP kinase (Fig. 8). Thus, the substrate specificity of CaMKP-N is high and similar to that of CaMKP (*26*). Since poly(Lys) (molecular weight 87K) enhanced the phosphatase activity of CaMKP-N, we examined the effect of polycations and basic proteins on the activity (Fig. 9). Like poly(Lys)(87K), poly(Lys) (9.6K) activated CaMKP-N. Histone and protamine activated CaMKP-N, but myelin basic protein, poly(Arg), and poly(Glu,Lys,Tyr) did not activate CaMKP-N. The biological significance of these effects is not clear and needs further investigation.

Subcellular Distribution of CaMKP-N—To examine the localization of CaMKP-N, we constructed GFP-fusion proteins. The GFP-CaMKP-N fusion protein was transiently expressed in COS-7 cells and its localization was detected by fluorescence microscopy. CaMKP-N was clearly localized in the nucleus (Fig. 10). Previously, we showed CaMKP is localized in the cytosol (*15, 16*). Thus, CaMKP and CaMKP-N were localized in distinct regions in the cells. In contrast to the full-length CaMKP-N fusion protein, a C-terminal deletion mutant lacking the C-terminal region from amino acid residue 574 to the last amino acid was localized in the cytosol. Other mutants that were shorter than this deletion mutant were localized in the cytosol or throughout the cell, depending on their size (data not shown). Fusion to GFP

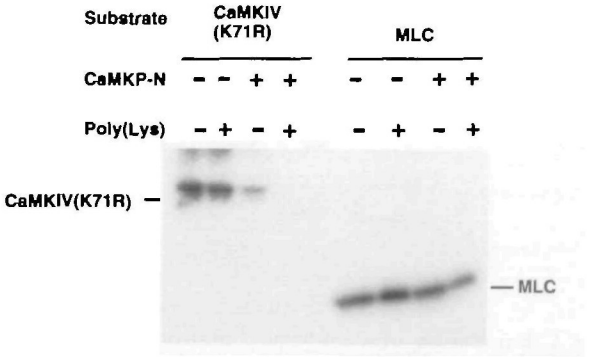

Fig. 7. **Dephosphorylation of CaMKIV and MLC by CaMKP-N.** CaMKIV(K71R) (7.35 nM), which had been phosphorylated by CaMK kinase α, and MLC (7.35 nM), which had been phosphorylated by PKC, were incubated at 30°C with or without CaMKP-N (0.1 μg/ml) in the presence or absence of 10 μg/ml poly(Lys), as described under "MATERIALS AND METHODS." After incubation for 30 min, the reaction was terminated, and aliquots were analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography.

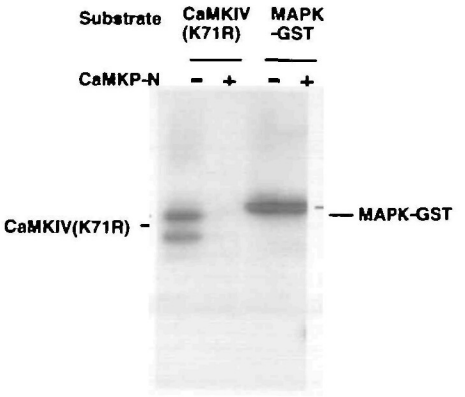

Fig. 8. **Dephosphorylation of CaMKIV and MAP kinase by CaMKP-N.** CaMKIV(K71R) (7.35 nM), which had been phosphorylated by CaMK kinase α, and MAP kinase-GST (7.35 nM), which had been phosphorylated by activated MEK1, were incubated at 30°C with or without CaMKP-N (1.0 μg/ml) in the presence or absence of 10 μg/ml poly(Lys), as described under "MATERIALS AND METHODS." After incubation for 30 min, the reaction was terminated, and aliquots were analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography.

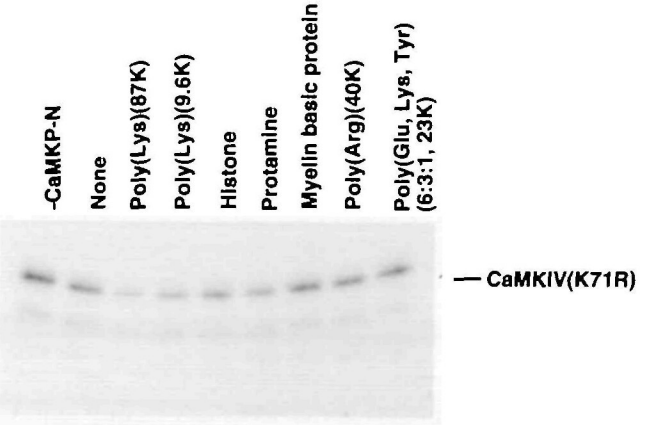

Fig. 9. **Activation of CaMKP-N by polycations and basic proteins.** CaMKIV(K71R) (7.35 nM), which had been phosphorylated by CaMK kinase α, was incubated at 30°C with or without CaMKP-N (1.0 μg/ml) in the presence or absence of 10 μg/ml poly(Lys), as described under "MATERIALS AND METHODS," except that 10 μg/ml poly(Lys) was replaced by the indicated polycations or basic proteins of 10 μg/ml. After incubation for 30 min, the reaction was terminated, and aliquots were analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography.

did not cause the nuclear localization, since the T7-tagged CaMKP-N was also localized in the nucleus (data not shown). Thus, the C-terminal region contained a nuclear localization signal, although further studies are necessary for the precise identification of the sequence.

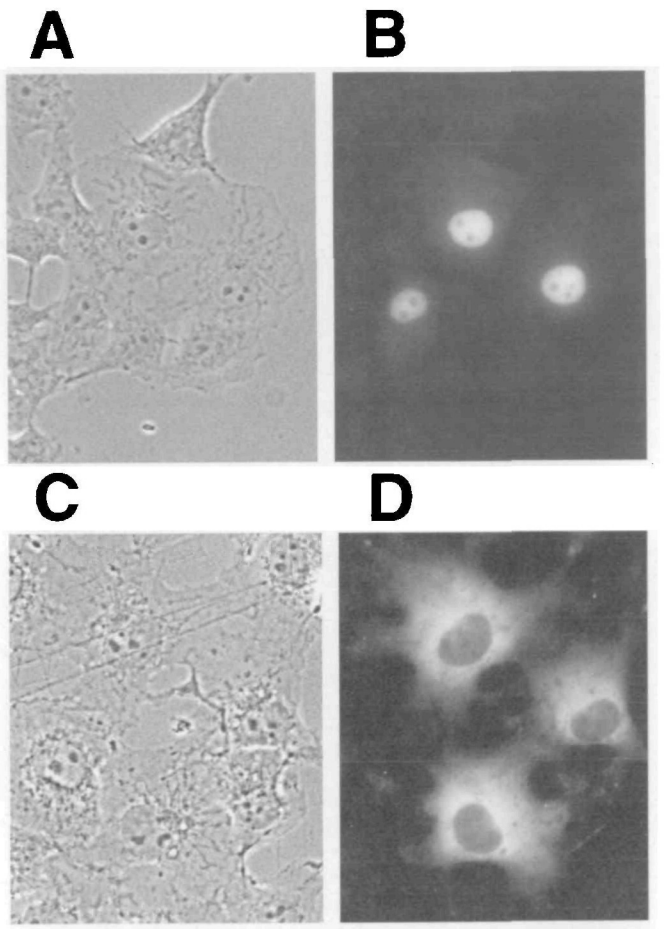

Fig. 10. **Subcellular localization of CaMKP-N and the deletion mutant.** COS-7 cells transfected with pEGFP-CaMKP-N (A and B) or pEGFP-CaMKP-N(1-573) (C and D) were visualized by GFP fluorescence. Phase-contrast images of the cells are shown in panel A and C.

DISCUSSION

Previously, we found a protein phosphatase, CaMKP, which is highly specific to CaMKs. In this study, we identified a CaMKP-related protein, CaMKP-N. The amino acid sequences of CaMKP and CaMKP-N showed a significant similarity, and their enzymatic properties resembled each other. CaMKP-N and CaMKP are Mn^{2+}-dependent, poly-(Lys)-stimulated protein phosphatases that belong to the PPM family but are also distantly related to other PPM proteins (Fig. 3). The most closely related protein is PP2Cδ, which was recently identified (*33*). Interestingly, PP2Cδ also exhibits a preferential requirement for Mn^{2+}, although the PPM family is characterized by Mn^{2+} or Mg^{2+}-dependent phosphatase activity (*34*). The crystal structure of PP2Cα (*35*) indicates that the conserved residues between CaMKP-N, CaMKP, and other PPM members are important residues for the structure and activity (Fig. 2).

The human genome sequence has been almost completely determined (*36, 37*). Using the draft sequences of the human genome from the public sequence database, the gene organization of CaMKP-N was constructed (Fig. 11). The gene of human CaMKP-N was mapped to Chromosome 17 and consisted of seven exons. The organization of the exons of CaMKP-N is similar to that of CaMKP (Fig. 1), although the lengths and sequences of their introns are different. The number and length of exons and introns of CaMKP-N and CaMKP are different from those of PP2Cα and other PP2C members (data not shown). These observations indicate that CaMKP-N and CaMKP are closely related to each other.

Although the enzymatic properties of CaMKP-N and CaMKP are similar, CaMKP-N is localized in the nucleus, and CaMKP is localized in the cytosol. These results, together with the previous reports regarding localization of CaMKs, indicate that CaMKP-N dephosphorylates CaMKIV and nuclear CaMKII, whereas CaMKP dephosphorylates CaMKI and cytosolic CaMKII. Further studies are necessary to determine the functions of CaMKP-N and CaMKP in living cells.

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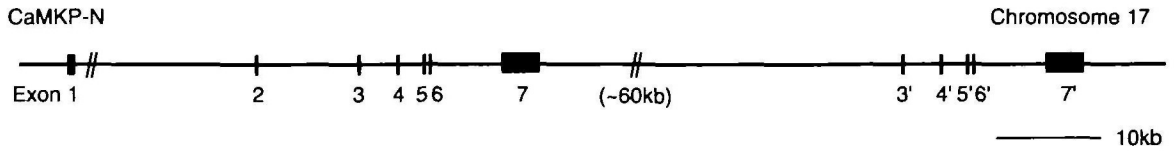

CaMKP-N

Exon1 1-597	...AATA/GTT------CAG/TAAT...	Exon2
Exon2 598-716	...GTGG/GTG------CAG/AGAT...	Exon3
Exon3 717-916	...AGAG/GTA------CAG/GACC...	Exon4
Exon4 917-1105	...GGAG/GTA------CAG/AGCT...	Exon5
Exon5 1106-1249	...AGAG/GTA------TAG/GATG...	Exon6
Exon6 1250-1343	...ATTG/GTA------CAG/GAGA...	Exon7
Exon7 1344-5035		

Fig. 11. **Gene organization of CaMKP-N.** The gene organization of CaMKP-N was constructed from the human genome draft sequence. The sequence between exon 1 and exon 2 is not completely determined. A region containing exon 3 through exon 7 was duplicated on the chromosome. The sequences of the exon–intron boundaries are shown. The exon–intron junctions are indicated by slashes.

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