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Subcellular distributions of rat CaM kinase phosphatase N and other members of the CaM kinase regulatory system

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Abstract

Ca^{2+}/Calmodulin-dependent protein kinase (CaM kinase) regulatory system is composed of multifunctional CaM kinases such as CaM kinases IV and I, upstream CaM kinases such as CaM kinase kinases α and β, which activate multifunctional CaM kinases, and CaM kinase phosphatases such as CaM kinase phosphatase and CaM kinase phosphatase N, which deactivate the activated multifunctional CaM kinases. To understand the combinations of CaM kinases I and IV, CaM kinase kinases α and β, and CaM kinase phosphatases, the locations of the enzymes in the cell were examined by immunocytochemical studies of cultured cells. The results indicate that CaM kinase I, CaM kinase kinase β, and CaM kinase phosphatase occur in the cytoplasm and that CaM kinase IV, CaM kinase kinase α (and CaM kinase kinase β in some cell types and tissues), and CaM kinase phosphatase N occur inside the cellular nucleus, suggesting that there are at least two different sets of CaM kinase regulatory systems, one consisting of CaM kinase I, CaM kinase kinase β, and CaM kinase phosphatase in the cytoplasm and the other consisting of CaM kinase IV, CaM kinase kinase α (and CaM kinase kinase β in some cell types and tissues), and CaM kinase phosphatase N in the nucleus.

Keywords: Ca^{2+} signaling, CaM kinase, CaM kinase cascade, CaM kinase phosphatase, rat brain, subcellular distribution.

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Multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) such as CaM kinases II, IV, and I play important roles in controlling a variety of cellular functions in response to an increase in intracellular Ca^{2+}, and the regulation of their activities is therefore very important (Hanson and Schulman 1992; Nairn and Picciotto 1994; Fujisawa 2001). Among the three CaM kinases, CaM kinase II is activated through phosphorylation by itself (Kwiatkowski *et al.* 1988; Ikeda *et al.* 1991; Katoh and Fujisawa 1991; Ishida *et al.* 1996), and the other two, CaM kinase IV (Okuno and Fujisawa 1993; Okuno *et al.* 1994; Tokumitsu *et al.* 1994) and CaM kinase I (Mochizuki *et al.* 1993; Lee and Edelman 1994), are activated through phosphorylation by upstream CaM kinase kinases. The activated CaM kinases are known to be deactivated through dephosphorylation by ubiquitous multifunctional protein phosphatases such as protein phosphatases 1, 2A and 2C. On the other hand, CaM kinase phosphatase (Kameshita *et al.* 1997; Ishida *et al.* 1998a) specifically dephosphorylates the phosphorylated multifunctional CaM kinases, thereby deactivating the activated CaM kinases (Ishida *et al.* 1998b). Immunocytochemical analysis of CaM kinase phosphatase revealed that it is localized only in the cytoplasm (Kitani *et al.* 1999; Nakamura *et al.* 2000). CaM kinase I, one of the targets of CaM kinase phosphatase, exists in the cytoplasm (Picciotto *et al.* 1995), but CaM kinase IV exists in the nuclei (Jensen *et al.* 1991; Nakamura *et al.* 1995), suggesting that CaM kinase IV is not an actual target of CaM kinase phosphatase. Recently, an isozyme of CaM kinase phosphatase, CaM kinase phosphatase N, was identified and, when the subcellular distribution of the recombinant green fluorescent protein (GFP)-fusion CaM kinase phosphatase N in the transfected cultured cells was examined, it existed inside the cellular nucleus (Takeuchi *et al.* 2001), suggesting the simple possibility that the phosphatase which deactivates the active phosphorylated CaM

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Abbreviations used: CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

kinase IV is not CaM kinase phosphatase but CaM kinase phosphatase N. With regard to the intracellular localization of upstream kinases of CaM kinases IV and I, there have been conflicting reports. Nakamura *et al.* (1996) have reported that CaM kinase kinase α is present in cellular nuclei of virtually all neurons in the brain, as studied by immunohistochemical analysis, but Sakagami *et al.* (2000) have reported that it is localized in the perikaryal cytoplasm. As to CaM kinase kinase β, Sakagami *et al.* (2000) have reported that it is localized in the perikaryal cytoplasm, but Nakamura *et al.* (2001) have reported that it is present not only in the cytoplasm but also in the cellular nucleus in varying relative concentrations, depending upon the regions of the brain. In the present study, cDNA for rat CaM kinase phosphatase N was cloned, its specific antibody was prepared, and its subcellular distribution was immunocytochemically studied. To resolve the discrepancies as to subcellular distributions of CaM kinase kinases α and β, their distributions were also immunocytochemically studied and compared with those of the other members of the CaM kinase cascade.

Materials and methods

Cloning and sequencing of rat CaM kinase phosphatase N

Because northern blot analysis of poly(A)$^+$ RNAs from rat cerebral cortex with human CaM kinase phosphatase N cDNA fragment (nucleotides 931–2422, GenBank/EMBL/DDBJ accession number AB028995; Takeuchi *et al.* 2001) as a probe showed a clear band of about 6.8 kb, an oligo(dT)-primed cDNA library constructed in λZAPII (Stratagene, La Jolla, CA, USA) using poly(A)$^+$ RNA, which was isolated from rat cerebral cortex by use of FastTrack 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual, was screened with the probe. Forty-five positive clones of 1.5–7.0 kbp were isolated from approximately 10^6 plaques, but all the clones lacked more than 300 nucleotides on the 5′ (upstream) end of the coding region, probably owing to steric hindrance of cDNA synthesis arising from a high GC content in this region. To obtain a cDNA containing the entire coding sequence of the enzyme, another cDNA library was constructed with a synthesized oligonucleotide, 5′-TCCCACATCGTAAACTCTCCC-TGGCTGC-3′ (complementary to nucleotides 952–979 in Fig. 1) as a primer, and screened with a DNA fragment corresponding to nucleotides 398–813 as a probe. Seven positive clones of 0.7–1.2 kbp were isolated from approximately 2×10^4 plaques, and all the clones of more than 1.0 kbp contained the 5′ end of the coding region.

The nucleotide sequences of the clones were determined by the dideoxynucleotide chain-termination method (Sanger *et al.* 1977), using a DNA sequencer model 4000 L (LI-COR).

Expression of CaM kinase phosphatase N cDNA in Sf9 cells

A cDNA fragment containing the entire coding sequence of rat CaM kinase phosphatase N, prepared by ligation of two cDNA fragments properly made from the clones obtained as described above, was introduced into a vaculovirus, AcNPV, using a Bac-To-Bac

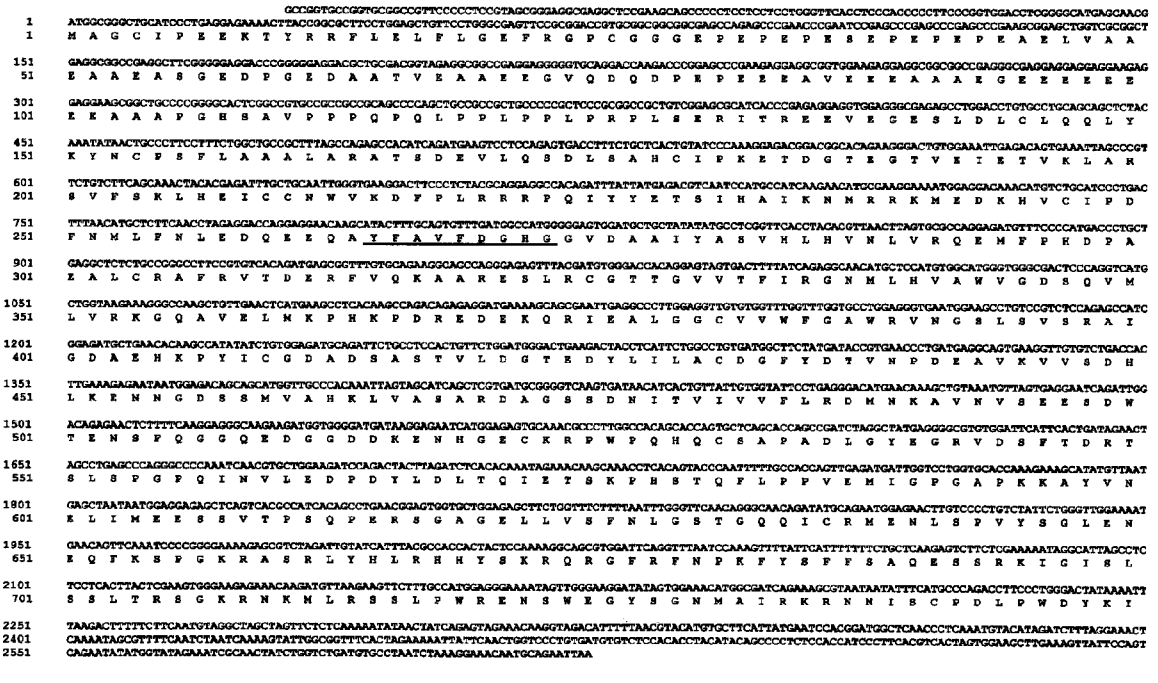

Fig. 1 Nucleotide sequence and deduced amino acid sequence of rat CaM kinase phosphatase N. Nucleotides are numbered beginning with the first nucleotide of the translational initiation codon. Amino acids are numbered beginning with the predicted amino-terminal residue. The underline indicates a protein phosphatase 2C motif (PROSITE entry no. PS01032).

Baculovirus Expression System with pFastBac 1 (Gibco-BRL/Life Technologies, Gaithersburg, MD, USA). Introduction of cDNA encoding His-tagged CaM kinase phosphatase N into AcNPV was carried out with the Bac-To-Bac System using pFastBacHT. Sf9 cells infected with the recombinant baculovirus were grown at 27°C in Grace's medium comprising 10% fetal bovine serum, 0.35 g/L $NaHCO_3$, 100 μg/mL kanamycin, and 2.6 g/L Bacto tryptose phosphate broth (Difco/Becton Dickinson and Company, Sparks, MD, USA) (pH 6.1). After 2 days, the cells were harvested, washed once with phosphate-buffered saline (PBS), suspended in approximately 10 volumes of 50 mM Tris–HCl buffer (pH 7.5 at 4°C) containing 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.1% Tween-40, 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 recoveries of the recombinant CaM kinase phosphatase N and His-tagged CaM kinase phosphatase N in the supernatant were approximately 10% and 50%, respectively. The His-tagged enzyme was purified using Ni-NTA chromatography (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Cell culture and transfection

A cDNA fragment encoding the desired protein was properly excised and inserted into a mammalian expression vector, pEGFP-N1 (Clontech, Palo Alto, CA, USA), before GFP coding sequence so that the expressed protein did not contain GFP. PC-12 cells were cultured on collagen type I-coated coverslips (Iwaki/Asahi Techno Glass, Tokyo, Japan) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 10% horse serum in a humidified incubator at 37°C under an atmosphere of 5% CO_2. Transfection of the cells with the expression vector was carried out using Targefect F-1 (Targeting Systems, Santee, CA, USA) or TransFast™ Transfection Reagent (Promega, Madison, WI, USA) according to the manufacture's instructions.

Immunocytochemical analysis

The cells grown on coverslips described above were rinsed with PBS, fixed with 3.7% formaldehyde in PBS for 30 min, washed three times in PBS, and then permeabilized with 0.3% Triton X-100 in PBS for 5 min. After washing in PBS, the cells were incubated with 3% normal goat serum (Cappel/ICN Biomedicals, Irvine, CA, USA) and 0.1% Triton X-100 in PBS for 1 h, incubated overnight at 4°C with 0.225 μg/mL antibody raised against CaM kinase phosphatase N, 0.325 μg/mL antibody against CaM kinase I, 0.114 μg/mL antibody against CaM kinase IV, 1.18 μg/mL antibody against CaM kinase kinase α (Okuno *et al.* 1996), 0.77 μg/mL antibody against CaM kinase kinase β (Kitani *et al.* 1997b), or 0.34 μg/mL antibody against CaM kinase phosphatase (Kitani *et al.* 1999), washed three times in 0.1% Triton X-100 in PBS, and then incubated with 1 μg/mL Alexa Fluor 488 goat anti-rabbit IgG (H + L), highly cross-adsorbed (Molecular Probes, Eugene, OR, USA) in 3% normal goat serum and 0.1% Triton X-100 in PBS for 2 h. After washing three times in 0.1% Triton X-100 in PBS, cell nuclei were visualized by staining with 0.2 μg/mL Hoechst 33258 (Polysciences, Warrington, PA, USA) for 5 min, followed by washing in three changes of PBS. The cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA), and then examined by fluorescence microscopy under a Zeiss Axiovert microscope (Carl Zeiss Japan, Tokyo, Japan) equipped with a DAPI/FITC/TRITC filter set (Chroma Technology, Brattleboro, UT, USA) using a Zeiss 40× Plan-Neofluor lens (Carl Zeiss). Microscopic data were analyzed by CELLscan system with CELLview image visualization and EPR image restoration systems (Scanalytics, Billerica, MA, USA).

Antibodies against CaM kinase phosphatase N, CaM kinase I, and CaM kinase IV were prepared by immunizing Japanese white rabbits with a peptide, CPDLPWDYKI, consisting of the carboxyl-terminal 10 amino acids of CaM kinase phosphatase N, a peptide, CVEPGSELPPAPPPSSRAMD, consisting of the carboxyl-terminal 20 amino acids of CaM kinase I, and a peptide, CEA-AAVGLGVPPQQDAILPEY, containing the carboxyl-terminal 20 amino acids of CaM kinase IV, respectively, and purified by affinity chromatography on the respective peptides-coupled columns, essentially as described for CaM kinase kinase α (Okuno *et al.* 1996) and CaM kinase phosphatase (Kitani *et al.* 1999).

Western blot analysis

Western blot analysis was performed essentially as described by Winston *et al.* (1987). Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on a 7.5% acrylamide gel, and then the protein bands were transferred to a polyvinylidene difluoride membrane (Fluorotrans, Pall Bio Support, Pall/Nippon Genetics, Tokyo, Japan). The membrane was blocked with 5% non-fat milk in PBS for 30 min at 24°C, and then incubated with antibodies to the desired protein in the blocking buffer at 4°C overnight, followed by incubation with 30 μg/mL goat anti-rabbit immunoglobulins (IgA + IgG + IgM) conjugated with peroxidase (Cappel/ICN Biomedicals) at 24°C for 1 h. Positive bands were detected with diaminobenzidine tetrahydrochloride and H_2O_2 in the presence of $CoCl_2$.

Results and discussion

Nucleotide and amino acid sequences of rat CaM kinase phosphatase N

A λZAPII cDNA library constructed from rat brain was screened by plaque hybridization with human CaM kinase phosphatase N cDNA. Figure 1 shows the nucleotide sequence of the predicted coding region and the deduced amino acid sequence. The cDNA sequence upstream of the nucleotide 400 has a high GC content. The coded protein consisted of 750 amino acids with a molecular weight of 83 438 and contained a protein phosphatase 2C motif (PROSITE entry no. PS01032). Figure 2 shows deduced amino acid sequence homologies between rat CaM kinase phosphatase N and CaM kinase phosphatase, and between rat and human CaM kinase phosphatase N. The overall homologies of rat CaM kinase phosphatase N with rat CaM kinase phosphatase and with the human homolog were 52 and 88%, respectively.

When cDNAs encoding CaM kinase phosphatase N and His-tagged CaM kinase phosphatase N were expressed in Sf9

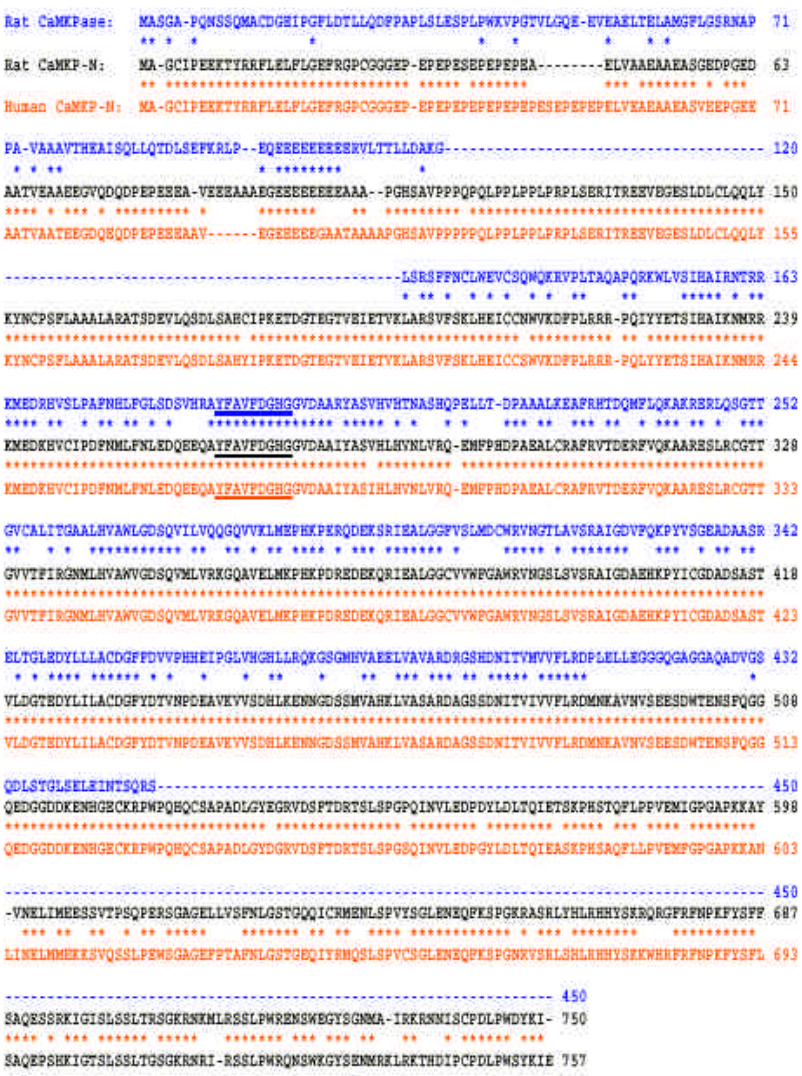

Fig. 2 Comparison of the deduced amino acid sequence of rat CaM kinase phosphatase N with those of the human homolog and rat CaM kinase phosphatase. The deduced amino acid sequences of rat CaM kinase phosphatase N (shown in black), rat CaM kinase phosphatase (blue; Kitani *et al.* 1999), and human CaM kinase phosphatase N (red; Takeuchi *et al.* 2001) are aligned for maximal homology, matching amino acids being indicated by asterisks. The underlines indicate the protein phosphatase 2C motif, as described in the legend for Fig. 1.

cells, approximately 90% of CaM kinase phosphatase N protein was found in the pellet but 50% of the His-tagged enzyme was recovered in the supernatant. Therefore, the CaM kinase phosphatase activity of the cloned protein was measured with the crude extract of Sf9 cells infected with vaculovirus carrying a cDNA of the His-tagged enzyme, as shown in Fig. 3. The crude extract showed high ability to dephosphorylate CaM kinase IV (K71R), which had been phosphorylated by CaM kinase kinase α on Thr196 (Kitani *et al.* 1997a), in the presence of 10 μg/mL polylysine. The crude extract of uninfected Sf9 cells showed weak CaM kinase IV-dephosphorylating activity but the activity was not significantly stimulated by polylysine. The marked activation by polylysine has been observed for rat CaM kinase phosphatase (Ishida *et al.* 1998a, 1998b; Kitani *et al.* 1999) and human CaM kinase phosphatase N (Takeuchi *et al.* 2001). Thus, the cloned cDNA does indeed encode rat CaM kinase phosphatase N.

Tissue distribution of CaM kinase phosphatase N

The tissue distribution of CaM kinase phosphatase N was examined by northern blot analysis (Fig. 4) and western blot analysis (Fig. 5). As shown in Fig. 4(a), an mRNA species of approximately 6.8 kb in brain and heart, the level being much higher in brain, and a species of 2.4 kb in testis were detected by northern blot analysis. No positive species were detected in spleen, lung, liver, skeletal muscle, and kidney under the experimental conditions used. The species of 6.8 kb were found in all brain regions examined, with the highest levels in hippocampus, thalamus, and midbrain, and the lowest level in spinal cord, as shown in Fig. 4(b).

When the tissue distribution was examined by western blot analysis with antibodies raised against the carboxyl-terminal 10 amino acids of CaM kinase phosphatase N as shown in Fig. 5, crude extracts of cerebral cortex, brain stem, and cerebellum showed a single immunoreactive band at the position coinciding with that of the transfected Sf9 cells, and

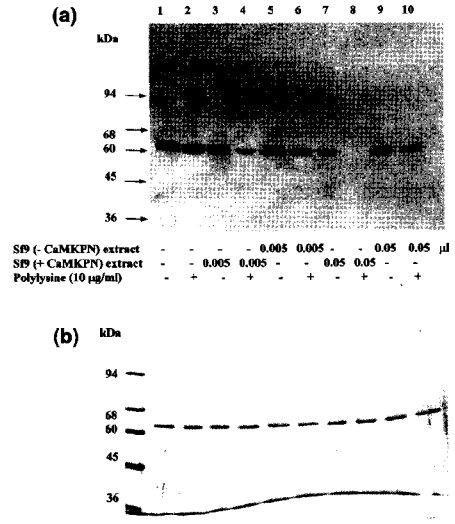

Fig. 3 Dephosphorylation of CaM kinase IV phosphorylated by CaM kinase kinase. Approximately 18 µg of CaM kinase IV (K71R), in which Lys71 (ATP-binding site) was replaced with arginine, expressed in Sf9 cells and purified as described previously (Kitani *et al.* 1997a), was phosphorylated at 30°C for 20 min with 0.5 µg of CaM kinase kinase α, expressed in *Escherichia coli* and purified as described previously (Kitani *et al.* 1997a), in 50 µL of a reaction mixture containing 50 mM Mops-NaOH (pH 7.0 at 30°C), 0.2 mM [γ-^{32}P]ATP (5000 cpm/pmol), 5 mM $Mg(CH_3COO)_2$, 2 mM dithiothreitol, 0.15 mM $CaCl_2$, and 9 µM calmodulin, expressed in *E. coli* and purified as described previously (Kitani *et al.* 1995). After the reaction was stopped by the addition of 6.5 mM EDTA, the mixture was applied to a small column of Sephadex G-50 (superfine) (0.9 mL) equilibrated with 10 mM Tris–HCl (pH 7.5 at 4°C), 50 mM NaCl, 0.05% Tween-40, and 0.1 mM dithiothreitol to remove ATP, Ca^{2+}, and EDTA. The column was eluted with the equilibration buffer by the spin column method (Sambrook *et al.* 1989), and the radioactive fractions eluted near the void volume were pooled. Aliquots [1.16 pmol of phosphorylated CaM kinase IV (K71R), 7026 cpm] were incubated at 30°C for 10 min with the indicated amounts of Sf9 cell extracts and/or 10 µg/mL poly-L-lysine (average molecular weight 87 000, Sigma, St Louis, MO, USA) in 10 µL of a mixture containing 50 mM Tris–HCl (pH 7.5 at 30°C), 2 mM $MnCl_2$, and 0.05% Tween-40, as indicated, and subjected to SDS–PAGE on a 7.5% acrylamide gel, followed by autoradiography (a) and protein staining with Coomassie brilliant blue (b). The Sf9 cell extracts were prepared from the Sf9 cells infected [Sf9 (+ CaMKPN)] or not infected [Sf9 (– CaMKPN)] with baculovirus carrying a cDNA encoding His-tagged CaM kinase phosphatase N, as described in Materials and methods. The protein concentrations of the Sf9 (– CaMKPN) and Sf9 (+ CaMKPN) extracts were 5.7 mg/mL and 3.4 mg/mL, respectively, as determined by a modified Lowry's method (Peterson 1977).

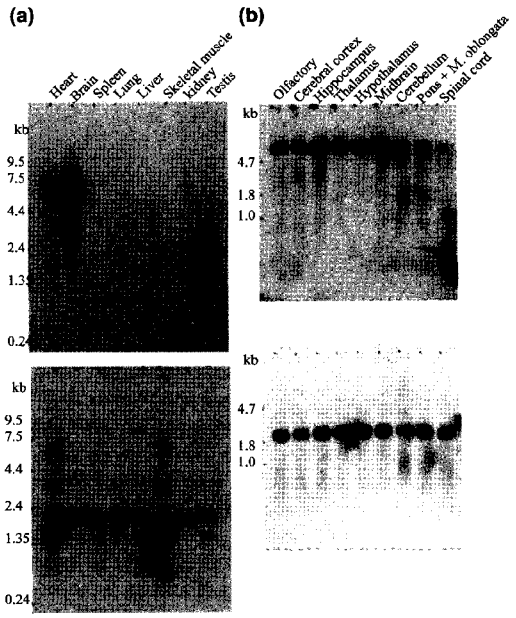

Fig. 4 Tissue distribution of CaM kinase phosphatase N on Northern blot analysis. Rat MTN blot (Clontech; a), on which 2 µg each of poly(A)$^+$ RNA from the indicated tissues had been blotted, and rat brain tissue blot (Seegene, Seoul, Korea; b), on which 20 µg each of total RNA isolated from the indicated brain regions had been blotted, were hybridized with a ^{32}P-labeled probe for CaM kinase phosphatase N (nucleotides 398–2273; upper panel) or ^{32}P-labeled human β-actin cDNA (Clontech; lower panel) in ExpressHyb hybridization solution (Clontech) at 68°C for approximately 2 h. The membrane filters were then washed with a solution consisting of 0.2 × SSC (saline–sodium citrate) and 0.1% SDS at 60°C. RNA sizes in kilobases are given on the left.

no other tissues examined showed a significant immunoreactive band at this position under the experimental conditions used. The results of northern and western blot analyses, taken together, suggest that CaM kinase phosphatase N is mainly expressed in brain, the contents varying from regions to regions as shown in Fig. 4(b), weakly expressed in heart, and not significantly expressed in other tissues.

The molecular weight of a single immunoreactive band observed in the transfected Sf9 cells, cerebral cortex, brain stem, and cerebellum were calculated from mobility on SDS–PAGE to be 123 000, which is much higher than that calculated from the deduced amino acid sequence of the enzyme, 83 438. To solve this discrepancy, His-tagged CaM kinase phosphatase N was expressed in Sf9 cells, and the molecular weight of His-tagged enzyme purified by Ni-NTA chromatography was estimated by mass spectrometry on a matrix-assisted laser desorption ionization-time of flight (TOF) (MALDI-TOF) mass spectrometer, REFLEX III (Bruker Daltonics, Bremen, Germany), using bovine serum albumin (molecular weights of 66 430 for monomer and 132 858 for dimer) for calibration standard. The estimated value was 87 669 (data not shown), which is closely in accord with the value, 87 539, calculated from the amino acid sequence of His-tagged CaM kinase phosphatase N,

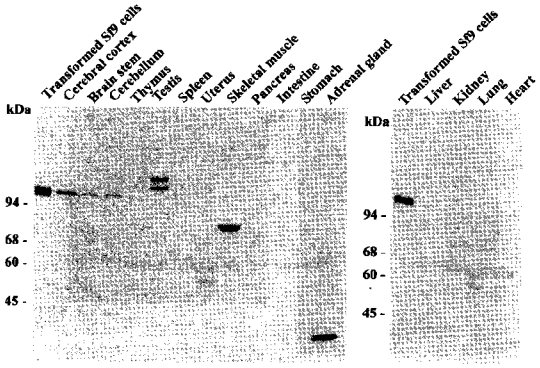

Fig. 5 Tissue distribution of CaM kinase phosphatase N on western blot analysis. Approximately 0.09 μg protein of the pellet of the homogenate of Sf9 cells transfected with baculovirus carrying a cDNA encoding CaM kinase phosphatase N (transfected Sf9 cells) and 50 μg protein of the crude extracts of the indicated rat tissues were subjected to western blot analysis with approximately 3.8 μg/mL antibodies against CaM kinase phosphatase N, as described in Materials and methods. The crude tissue extracts were prepared by homogenization of tissues obtained from 10-week-old female Wistar rats and testes from male rats with a Potter–Elvehjem homogenizer in 3 volumes of 20 mM HEPES-NaOH (pH 7.5 at 4°C) containing 1 mM dithiothreitol, 0.1% Triton X-100, and 20 μg/mL each of the microbial protease inhibitors (pepstatin A, leupeptin, antipain, and chymostatin), followed by centrifugation.

although its molecular weight estimated from mobility on SDS–PAGE was about 120 000, which strangely is smaller than that of untagged CaM kinase phosphatase N. These results indicate abnormal behavior of CaM kinase phosphatase N on SDS–PAGE.

Subcellular distributions of components of CaM kinase cascade

There have remained some ambiguities about the subcellular distributions of the components of CaM kinase cascade: (i) subcellular distribution of CaM kinase phosphatase N was examined with the recombinant GFP-fusion enzyme, but not with the enzyme itself; (ii) CaM kinase kinase α has been reported to occur in cellular nuclei of all neurons in the brain in one paper (Nakamura *et al.* 1996) but reported to occur in the perikaryal cytoplasm in another paper (Sakagami *et al.* 2000); and (iii) CaM kinase kinase β has been reported to occur in the perikaryal cytoplasm in one paper (Sakagami *et al.* 2000) but reported to occur not only in the cytoplasm but also in the cellular nucleus in varying relative concentrations, depending upon the regions of the brain in another paper (Nakamura *et al.* 2001). To define the actual combinations of terminal multifunctional CaM kinases such as CaM kinases I and IV, upstream protein kinases such as CaM kinase kinases α and β, and protein phosphatases such as CaM kinase phosphatase and CaM kinase phosphatase N, subcellular distributions of these components of CaM kinase cascade were studied (Fig. 6). CaM kinase I was observed in the cytoplasm of PC-12 cells, in agreement with earlier studies (Picciotto *et al.* 1995), and was not observed in the nucleus. CaM kinase IV, even when highly expressed in PC-12 cells, was localized in the nucleus and was not observed in the cytoplasm, suggesting that CaM kinase IV is very efficiently translocated from the cytoplasm to the nucleus. CaM kinase kinase α was observed in the nucleus of PC-12 cells and not significantly observed in the cytoplasm, in agreement with our earlier observation (Nakamura *et al.* 1996). Essentially identical results (data not shown) were also obtained with monoclonal antibody raised against CaM kinase kinase α which had been used for the study of CaM kinase kinase α localization by Sakagami *et al.* (2000). However, when CaM kinase kinase α was highly expressed in PC-12 cells by transfection with the cDNA, not the nucleus but the cytoplasm was strongly stained (data not shown), indicating that the rate of the translocation of CaM kinase kinase α into the nucleus is not so high. As to CaM kinase kinase β, the immunoreactivity was much higher in the cytoplasm than in the nucleus in PC-12 cells but a few granular immunofluorescent spots were observed inside the nucleus. Thus, CaM kinase kinase α was located almost exclusively in the nucleus of PC-12 cells, but CaM kinase kinase β was present mostly in the cytoplasm, although a little in the nucleus of PC-12 cells. As Nakamura *et al.* (2001) have demonstrated that CaM kinase kinase β is present both in the cytoplasm and nucleus of most neurons but is present only in the cytoplasm of some large neurons in the mesencephalic trigeminal nucleus, lateral vestibular nucleus, or gigant cellular reticular formation, the extent of the translocation of CaM kinase kinase β from the cytoplasm to nucleus may differ from one cell type and tissue to another. CaM kinase phosphatase exhibited a distinctly cytoplasmic location with no immunofluorescence detected in the nucleus, in agreement with previous reports (Kitani *et al.* 1999; Nakamura *et al.* 2000). As cultured cells producing CaM kinase phosphatase N endogenously were not available, the location of the enzyme was studied with the transfected cells. CaM kinase phosphatase N, highly expressed in the transfected PC-12 cells, was found exclusively in the nucleus and not found in the cytoplasm, indicating highly efficient nuclear translocation of CaM kinase phosphatase N. Similar results (data not shown) were also obtained with the transfected COS-7 cells. Nuclear localization of CaM kinase phosphatase N has been suggested by GFP-fluorescence study of COS-7 cells transfected with cDNA for CaM kinase phosphatase N fused to GFP (Takeuchi *et al.* 2001).

In conclusion, CaM kinase IV and CaM kinase phosphatase N are present exclusively in the cellular nucleus, presumably due to the presence of efficient nuclear localization signals in their amino acid sequences. GFP-fusion CaM kinase phosphatase N lacking the carboxyl-terminal region is

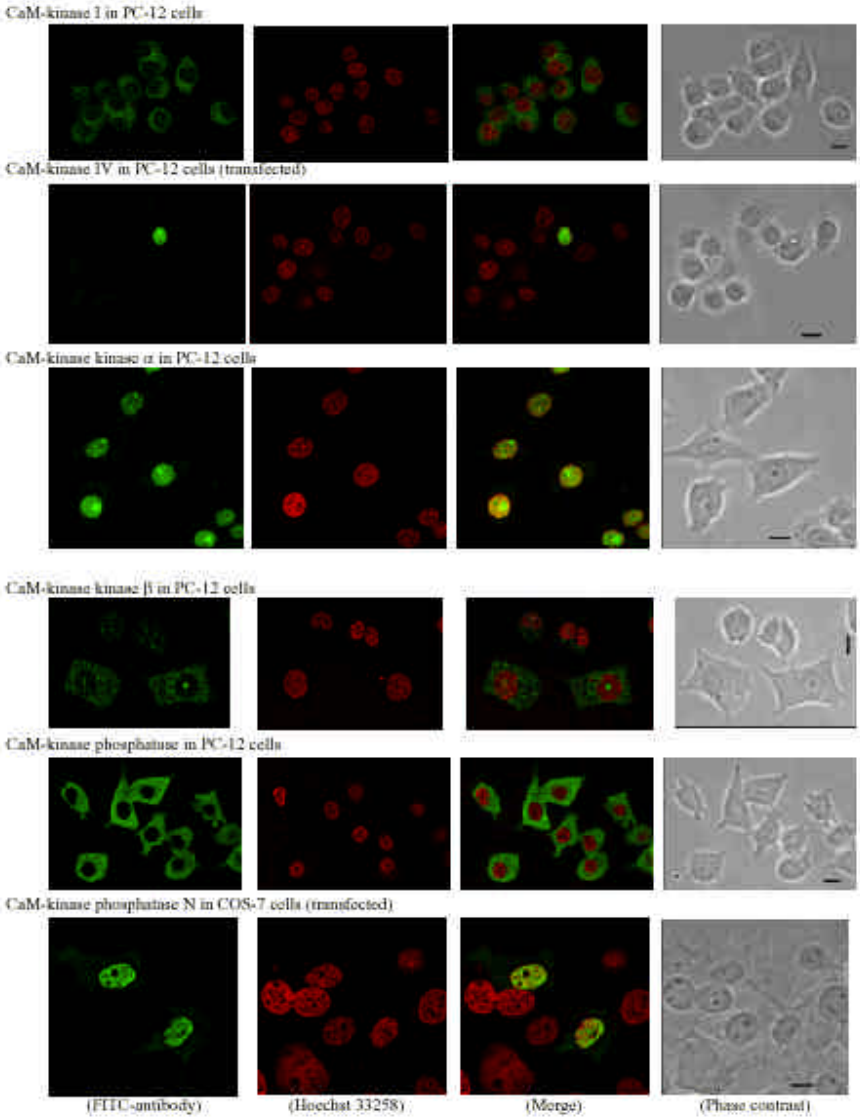

Fig. 6 Subcellular distributions of CaM kinase phosphatase N and other components of the CaM kinase cascade. PC-12 cells were stained by means of indirect immunofluorescence with antibodies against CaM kinase I, CaM kinase kinase α or β or CaM kinase phosphatase, and PC-12 cells, which were transfected with mammalian expression vector carrying CaM kinase IV cDNA or CaM kinase phosphatase N cDNA, were stained with the respective antibodies, as described in Materials and methods. The nuclei of the cells were stained with Hoechst 33258. A proper plane of the images was selected and the images of Alexa Fluor 488 (the left row of panels) and Hoechst 33258 (the next row) were merged together (the third row) using MetaMorph Imaging System (Universal Imaging Corporation). Phase-contrast images of the cells are shown in the right row of panels. Bar = 10 μm.

not found in the nucleus but in the cytoplasm (Takeuchi *et al.* 2001), indicating the presence of the signals in the carboxyl-terminal region. CaM kinase kinase α is also present in the cellular nucleus, but its nuclear localization signal or the machinery for nuclear translocation of the enzyme of PC-12 cells may be too weak to translocate large amounts of the enzyme expressed in the transfected cells from the cytoplasm to nucleus, in contrast with the cases of CaM kinase IV and CaM kinase phosphatase N. On the other hand, CaM kinase I and CaM kinase phosphatase are present exclusively in the cytoplasm, presumably due to the absence of nuclear localization signals. As to CaM kinase kinase β, it was present mostly in the cytoplasm but a little in the nucleus in PC-12 cells in the present study, suggesting that it can be translocated from the cytoplasm to the nucleus. This result together with the observation that CaM kinase kinase β is present only in the cytoplasm of some neurons but present both in the cytoplasm and nucleus of other neurons

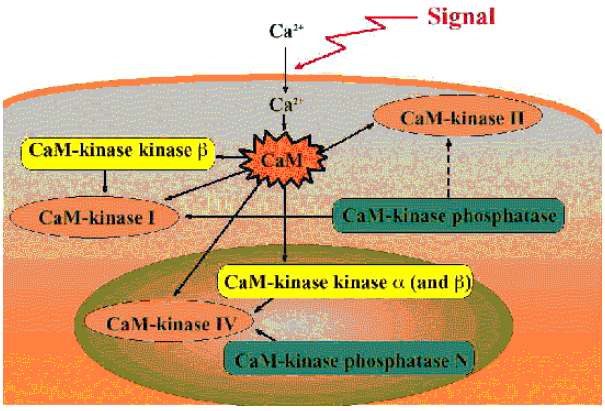

Fig. 7 A proposed pathway of CaM kinase cascade. See text.

(Nakamura *et al.* 2001) indicates that some cells have the machinery for nuclear translocation of CaM kinase kinase β but other cells do not. On the basis of the above result, it can be concluded that there are at least two different sets of the CaM kinase regulatory systems, one consisting of CaM kinase I, CaM kinase kinase β, and CaM kinase phosphatase in the cytoplasm and the other consisting of CaM kinase IV, CaM kinase kinase α (and CaM kinase kinase β in some cell types and tissues), and CaM kinase phosphatase N, as summarized in Fig. 7. As CaM kinase II is known to exist mostly in the cytoplasm and post-synaptic membrane of neuronal cells, the enzyme activated upon autophosphorylation may be a target of CaM kinase phosphatase.

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