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Phosphorylation and activation of nuclear Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP-N/PPM1E) by Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI)

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ABSTRACT

Nuclear Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP-N/PPM1E) is an enzyme that dephosphorylates and downregulates multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) as well as AMP-dependent protein kinase. In our previous study, we found that zebrafish CaMKP-N (zCaMKP-N) underwent proteolytic processing and translocated to cytosol in a proteasome inhibitor-sensitive manner. In the present study, we found that zCaMKP-N is regulated by phosphorylation at Ser-480. When zCaMKP-N was incubated with the activated CaMKI, time-dependent phosphorylation of the enzyme was observed. This phosphorylation was significantly reduced when Ser-480 was replaced by Ala, suggesting that CaMKI phosphorylates Ser-480 of zCaMKP-N. Phosphorylation-mimic mutants, S480D and S480E, showed higher phosphatase activities than those of wild type and S480A mutant in solution-based phosphatase assay using various substrates. Furthermore, autophosphorylation of CaMKII after ionomycin treatment was more severely attenuated in Neuro2a cells when CaMKII was cotransfected with the phosphorylation-mimic mutant of zCaMKP-N than with the wild-type or non-phosphorylatable zCaMKP-N. These results strongly suggest that phosphorylation of zCaMKP-N at Ser-480 by CaMKI activates CaMKP-N catalytic activity and thereby downregulates multifunctional CaMKs in the cytosol.

1. Introduction

Nuclear Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKP-N/PPM1E) and its cytosolic homolog CaMKP/PPM1F are unique protein phosphatases that specifically dephosphorylate and regulate multifunctional CaMKs [1–5]. Recently, CaMKP-N and CaMKP were reported to dephosphorylate AMP-dependent protein kinase (AMPK) as well as CaMKs [6]. Therefore, these phosphatases may play key roles as signal regulators not only in neuronal systems but also in metabolic pathways responsible for energy homeostasis. CaMKP was found to be activated by phosphorylation with CaMKII [7], and stimulated by the addition of polycations such as poly-L-lysine [8]. However, detailed mechanisms for regulation of CaMKP-N activity *in vivo* still remain to be elucidated.

Recently, using human and zebrafish CaMKP-N expressed in Neuro2a cells as a model system, we found that the proteolytic processing occurred in the C-terminal region of CaMKP-N, and that proteasome inhibitors markedly suppressed the processing of CaMKP-N in Neuro2a cells [9]. Using MG-132, we found that the proteolytic processing changed the subcellular localization of CaMKP-N from the nucleus to the cytosol. In addition to our previous report that CaMKII phosphorylates and activates CaMKP [7], the observation that the proteolytic processing of CaMKP-N regulates its subcellular localization led us to the notion that the truncated form of CaMKP-N may be phosphorylated and regulated by cytosolic CaMKs.

In the present study, we found that Ser-480 of zebrafish CaMKP-N (zCaMKP-N) was phosphorylated by CaMKI. Phosphatase activities of S480D and S480E mutants, in which Ser-480 was replaced with Asp or Glu to mimic phosphorylated state, showed higher phosphatase activities than those of wild type and S480A mutant both *in vitro* and *in vivo*. We provide the evidence to show that specific phosphorylation of zCaMKP-N at Ser-480 by CaMKI upregulates its phosphatase activity and may cause downregulation of multifunctional CaMKs in the cytosol.

Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKP-N, nuclear Ca^{2+}/calmodulin-dependent protein kinase phosphatase; MUP, 4-methylumbelliferyl phosphate; NLS, nuclear localization signal.

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2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (111 TBq/mmol) was purchased from PerkinElmer. ATP, anti-actin antibody, Cy3-labeled anti-mouse IgG and 4-methylumbelliferyl phosphate (MUP) were obtained from Sigma Chemicals. Goat anti-mouse IgG and Goat anti-rabbit IgG conjugated with horseradish peroxidase were obtained from Pierce. MG-132 and Epoxomicin were purchased from PEPTIDE Institute. Recombinant rat CaM [10], rat CaMKIα [11], mouse CaMKKα [12], zebrafish CaMKP-N and its mutants [9,13] were expressed in *Escherichia coli*, and purified as described. Anti-GFP and anti-His_6 antibodies were from Roche and Wako Pure Chemical Industries, respectively. Anti-phospho-CaMKI [14] and anti-CaMKP-N [9] antibodies were obtained according to the methods in the cited reports. Anti-phospho-CaMKII(Thr-286) was purchased from Thermo SCIENTIFIC.

2.2. Construction of plasmids

Expression vectors for full-length of wild-type or phosphatase-dead CaMKP-N, pETzCaMKP-N(WT) or pETzCaMKP-N(D188A), were generated as described previously [9]. Point mutants of putative phosphorylation sites (S38A, T245A, S366A, S480A) and phosphorylation-mimic mutants on Ser-480 (S480D and S480E) were generated by inverse PCR method [15] using pETzCaMKP-N(1-502) (WT or D188A) [9] as a template. All of the cDNAs described above were subcloned into the multicloning site of pET23a to generate His_6-fused protein at their C-terminal ends.

For expression of zCaMKP-N and its mutants in mammalian cells, we constructed pcDNA-zCaMKP-N(WT) [9], pcDNA-zCaMKP-N(D188A) [9], pEGFP-zCaMKP-N [9] and pcDNA-CaMKI [16] as described. An expression vector for rat CaMKIIα (pcDNA-CaMKII) was generated by PCR using rat brain cDNA library as a template. pcDNA-zCaMKP-N(1-502) and its mutants on Ser-480 (S480A, S480D, S480E) were prepared by inverse PCR [15] using pcDNA-zCaMKP-N(WT) and pcDNA-zCaMKP-N(1-502) as templates, respectively.

2.3. Cell culture and transfection

Mouse neuroblastoma Neuro2a cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% heat-inactivated fetal calf serum (FCS) at 37 °C in a humidified incubator with a 5% CO_2/95% air atmosphere. Transfection of Neuro2a cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. Treatment of Neuro2a cells with proteasome inhibitors

Transfected cells were cultured for 12 h and then treated with 0.1% DMSO (negative control), 10 μM MG-132 or 1 μM Epoxomicin in DMEM containing 10% FCS for 12 h. The treated cells were washed with PBS, lysed with SDS–PAGE sample buffer and analyzed by Western blotting.

2.5. SDS–PAGE and Western blotting

SDS–PAGE was carried out essentially according to the method of Laemmli [17] on a slab gel consisting of a 10% separation gel and a 3% stacking gel. Western blotting was carried out as described previously [18].

2.6. Phosphorylation of CaMKP-N by CaMKI

For preparation of activated CaMKI, 100 ng of CaMKI was incubated in the reaction mixture (10 μl) consisting of 50 mM Hepes-NaOH (pH 7.5), 10 mM $Mg(CH_3COOH)_2$, 0.1 mM EGTA, 5 ng CaMKK, 1 mM $CaCl_2$, 1 μM CaM and 100 μM ATP at 30 °C for 30 min, and the reaction was stopped by the addition of 1 μl of 20 mM EGTA. Recombinant CaMKP-N(1-502)(D188A) (200 ng) were incubated with or without CaMKI (2 ng) or phosphorylated CaMKI by CaMKK (2 ng) in the standard phosphorylation mixture (10 μl) containing 100 μM [γ-^{32}P]ATP. After incubation at 30 °C for the indicated time, equal volume of 2× SDS sample buffer was added to the phosphorylation mixture to stop the reaction. Phosphorylated proteins were then resolved on SDS–PAGE and visualized by autoradiography. [^{32}P]phosphate incorporation into CaMKP-N was quantified by a liquid scintillation counter.

2.7. Protein phosphatase assay using MUP as a substrate

The protein phosphatase assay was carried out at 30 °C for 10 min in a reaction mixture (100 μl) containing 50 mM Tris–HCl (pH 8.0), 10 mM $MnCl_2$, 20 mM dithiothreitol, 0.1 mM EGTA, 0.01% Tween 20, 25 μM MUP and 3 μg of CaMKP-N. The reaction was started by the addition of CaMKP-N and terminated by the addition of 25 μl of 500 mM EDTA. The 4-methylumbelliferone present in the reaction mixture was measured in a CytoFluor 4000TC fluorescence microplate reader (ABI) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

2.8. Protein phosphatase assay using phosphopeptide as a substrate

Protein phosphatase assay was carried out using phosphopeptide pp10 (YGGMHRQETpVDC), which contains an amino acid sequence around the autophosphorylation site of CaMKII, as a substrate [11]. Phosphatase assay was carried out in the reaction mixture (50 μl) containing 50 mM Tris–HCl (pH 8.0), 2 mM $MnCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 20 μM pp10 and an appropriate amount of CaMKP-N. The reaction was started by adding CaMKP-N and incubated at 30 °C for 6 min. The inorganic phosphate released in the mixture was determined by malachite green assay [19].

2.9. Protein phosphatase assay using phosphoprotein as a substrate

Protein phosphatase assay was carried out using CaMKI as a phosphoprotein substrate. Recombinant rat CaMKIα(K49R) (1 μg) was phosphorylated by CaMKK (50 ng) at 30 °C for 30 min in a reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 1 μM CaM, 1 mM $CaCl_2$ and 100 μM ATP, and the reaction stopped by adding EGTA to the final concentration of 2 mM. Dephosphorylation of phospho-CaMKI was carried out at 30 °C for 10 min in a reaction mixture containing 50 mM Tris–HCl (pH 8.0), 2 mM $MnCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 1 mM dithiothreitol, 100 ng phospho-CaMKI and 2 ng CaMKP-N. The reaction was started by the addition of CaMKP-N and terminated by the addition of an equal volume of 2× SDS–PAGE sample buffer. The sample was then subjected to SDS–PAGE and analyzed by Western blotting using phospho-CaMKI antibody.

2.10. Immunocytochemistry

Transfected cells were cultured on cover glasses and treated with 3.7% formaldehyde in PBS for 20 min. After rinsing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After treatment with 1% bovine serum albumin in PBS, the cells were incubated with an anti-Myc antibody diluted 1:1000 in PBS containing 1% bovine serum albumin at 4 °C for

16 h, followed by incubation with Cy3-labeled anti-mouse IgG at room temperature for 2 h. The nuclei were counterstained with DAPI. The stained cells were observed using a confocal laser-scanning microscope (FM1000-D; OLYMPUS).

3. Results

3.1. Subcellular localization of CaMKP-N and its processing product

In our previous study, we reported that the majority of naturally occurring CaMKP-N as well as overexpressed CaMKP-N in various cultured cell lines readily undergoes proteolysis at its C-terminal region [9]. The limited proteolysis is effectively inhibited by the proteasome inhibitors MG-132 and Epoxomicin, suggesting that the ubiquitin–proteasome pathway is involved in this processing. Actually, almost all of the GFP-fused zCaMKP-N expressed in Neuro2a cells existed as a truncated form (Fig. 1A, arrowhead). When the cells were treated with proteasome inhibitors, however, the proteolytic processing was markedly suppressed (Fig. 1A, arrow). Simultaneously, we observed the subcellular localization of GFP-zCaMKP-N by confocal laser microscopy. In control Neuro2a cells, where the processing should occur, GFP-zCaMKP-N was detected in both the cytosol and the nucleus (Fig. 1B, a–d). When the Neuro2a cells were treated with MG-132, where the processing should be suppressed, GFP-zCaMKP-N was localized only in the nucleus (Fig. 1B, e–h). These results suggested that full-length zCaMKP-N containing the NLS is localized in the nucleus, whereas the truncated form of zCaMKP-N that lacks the C-terminal NLS motif exhibits widespread distribution in the cytoplasm.

3.2. Phosphorylation of CaMKP-N by CaMKI

In addition to our previous report that CaMKII phosphorylates and activates CaMKP [7], the observation that the proteolytic processing of CaMKP-N regulates its subcellular localization led us to the notion that the truncated form of CaMKP-N may be phosphorylated and regulated by cytosolic CaMKs. To check this possibility, we examined whether the proteolyzed zCaMKP-N, which was localized in the cytoplasm, could be phosphorylated by cytosolic CaMKI. In a previous paper [13], we reported that the region consisting of amino acid residues 575–587 of zCaMKP-N functions as an NLS to localize zCaMKP-N only in nuclei, and that a mutant of zCaMKP-N lacking the NLS is distributed throughout the cells. In this study, we used a deletion mutant of zCaMKP-N lacking the NLS, zCaMKP-N(1-502), as a substrate for CaMKI. As shown in Fig. 2A and B, not only the recombinant CaMKP-N(1-502) but also the full-length CaMKP-N(FL) were phosphorylated by CaMKI only in the presence of CaMKK. A time course of phosphate incorporation into CaMKP-N by the activated CaMKI revealed that zCaMKP-N(1-502) was more efficiently phosphorylated than full-length CaMKP-N(FL) (~0.5 mol Pi/mol zCaMKP-N(1-502) vs. ~0.4 mol Pi/mol zCaMKP-N(FL)).

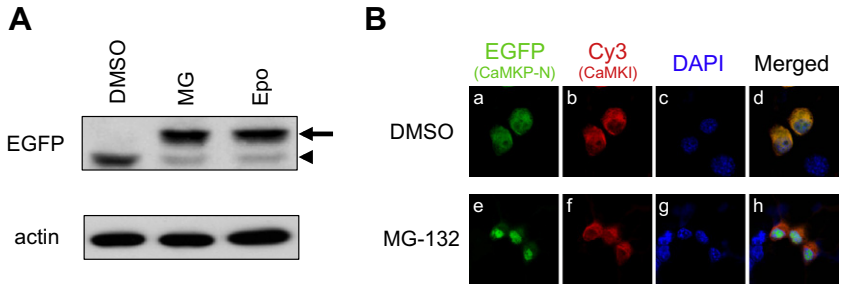

Fig. 1. Subcellular localization of proteolytic product of CaMKP-N. (A) Neuro2a cells transfected with pEGFP-zCaMKP-N were cultured for 12 h and then treated with or without the proteasome inhibitors MG-132 (MG, 10 μM) or Epoxomicin (Epo, 1 μM) for 12 h. The cells were lysed with SDS–PAGE sample buffer and the cell lysates (20 μg) were analyzed by Western blotting with anti-GFP or anti-actin antibodies. An arrow and an arrowhead indicate full-length zCaMKP-N and its proteolytic fragment, respectively. (B) Neuro2a cells co-transfected with pEGFP-zCaMKP-N and pcDNA-CaMKI were cultured for 12 h and then treated with 0.1% DMSO or 10 μM MG-132 for 12 h. Transiently expressed EGFP-zCaMKP-N and CaMKI-myc were visualized by means of indirect immunofluorescence with an anti-myc antibody (Cy3) using confocal laser scanning microscopy. The nuclei were counterstained by DAPI.

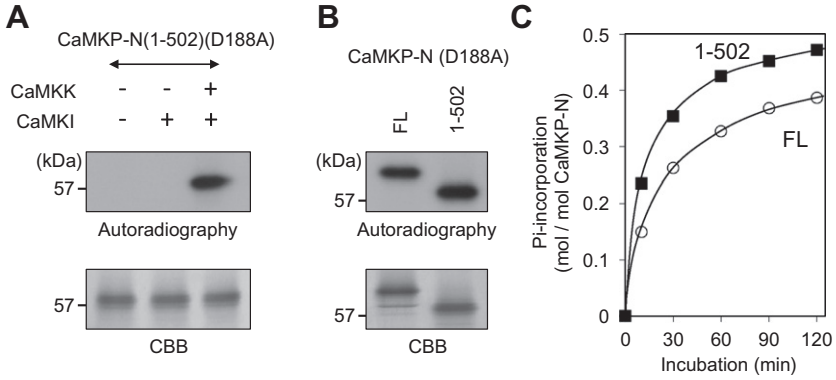

Fig. 2. Phosphorylation of CaMKP-N by CaMKI. (A) Recombinant zCaMKP-N(1-502)(D188A) (200 ng) was incubated with phosphorylated CaMKI (2 ng) by CaMKK in the standard phosphorylation mixture (10 μl) containing 100 μM [γ-^{32}P]ATP. After 30-min of incubation at 30 °C, the reaction was stopped by the addition of 2× SDS–PAGE sample buffer, subjected to 10% SDS–PAGE and visualized by autoradiography (upper panel) or stained with Coomassie brilliant blue (lower panel). (B) Phosphatase-dead mutant of zCaMKP-N(FL) or zCaMKP-N(1-502) (200 ng) was incubated with phosphorylated CaMKI (2 ng) and visualized as in (A). (C) Time course of [^{32}P]phosphate incorporation into zCaMKP-N. zCaMKP-N(FL)(D188A) and zCaMKP-N(1-502)(D188A) were phosphorylated by phospho-CaMKI as in (A) for the indicated time periods. [^{32}P]phosphate incorporation into zCaMKP-N was quantified by a liquid scintillation counter.

3.3. Identification of a phosphorylation site by CaMKI

Next, we tried to identify the phosphorylation site in CaMKP-N(1-502). It is reported that substrate recognition sequence for mammalian CaMKI is Arg-X-X-Ser/Thr [20]. There are five possible sites with consensus phosphorylation sequence by CaMKI in zCaMKP-N(1-502); Ser-38, Thr-245, Thr-256, Ser-366 and Ser-480. Among these, Thr-256 is not conserved in other CaMKP-N homologs, while other sites are highly conserved. Therefore, we prepared point mutants in which Ser-38, Thr-245, Ser-366 or Ser-480 was replaced with Ala. When these point mutants were phosphorylated with activated CaMKI, only S480A mutant showed phosphate incorporation less than 10% of that of wild-type CaMKP-N (Fig. 3A and B), suggesting that the major phosphorylation site in zCaMKP-N for CaMKI is Ser-480. Since amino acid sequences of CaMKP-N surrounding the phosphorylation site are highly conserved among zebrafish, human and rat CaMKP-N (Fig. 2C), the corresponding Ser residues in them may also be phosphorylated by CaMKI. In fact, the phosphorylation level of human CaMKP-N mutant in which the corresponding Ser residue was replaced with Ala (S550A) was significantly reduced as compared to the wild type when they were phosphorylated by CaMKI (data not shown).

3.4. Phosphatase activity of phosphorylation-mimic mutants of CaMKP-N

We next examined whether the phosphorylation of CaMKP-N affected its catalytic activity. To examine whether phosphorylation at Ser-480 was involved in the regulation of phosphatase activity, we carried out phosphatase assays using zCaMKP-N(1-502) harboring an Asp or a Glu substitution at Ser-480 (S480D or S480E), which mimic phosphorylation by CaMKI. Phosphatase activities of these mutants were measured by the solution-based protein phosphatase assay using phosphopeptide pp10 (Fig. 4A), fluorogenic substrate MUP (Fig. 4B) and protein substrate phospho-CaMKI (Fig. 4C and D). In each case, zCaMKP-N(1-502)(S480A) showed phosphatase activity comparable to the wild type enzyme. In contrast, zCaMKP-N(1-502)(S480D) and zCaMKP-N(1-502)(S480E) turned out to have significantly higher activities than those of wild-type and S480A mutant. We also carried out kinetic analysis of these phosphatases using pp10 as a substrate. As shown in Table 1, S480D and S480E mutants showed significantly higher V_{max} values than that of wild type enzyme without significant changes in K_m values.

To confirm whether zCaMKP-N is activated by phosphorylation at Ser-480 in Neuro2a cells, they were transiently transfected with CaMKII or with both CaMKII and zCaMKP-N mutants, and stimulated by ionomycin, a calcium ionophore. The phosphorylation level of CaMKII was analyzed by Western blotting using an anti-phospho-CaMKII antibody. As shown in Fig. 4E, the phosphorylation level of CaMKII was markedly increased in cells transfected with CaMKII alone in response to ionomycin stimulation (Fig. 4E, lanes 3 and 4). In cells cotransfected with wild type or S480A mutant of zCaMKP-N, however, autophosphorylation of CaMKII was significantly suppressed (Fig. 4E, lanes 4–6). When the cells cotransfected with CaMKII and S480D or S480E mutants were trea-

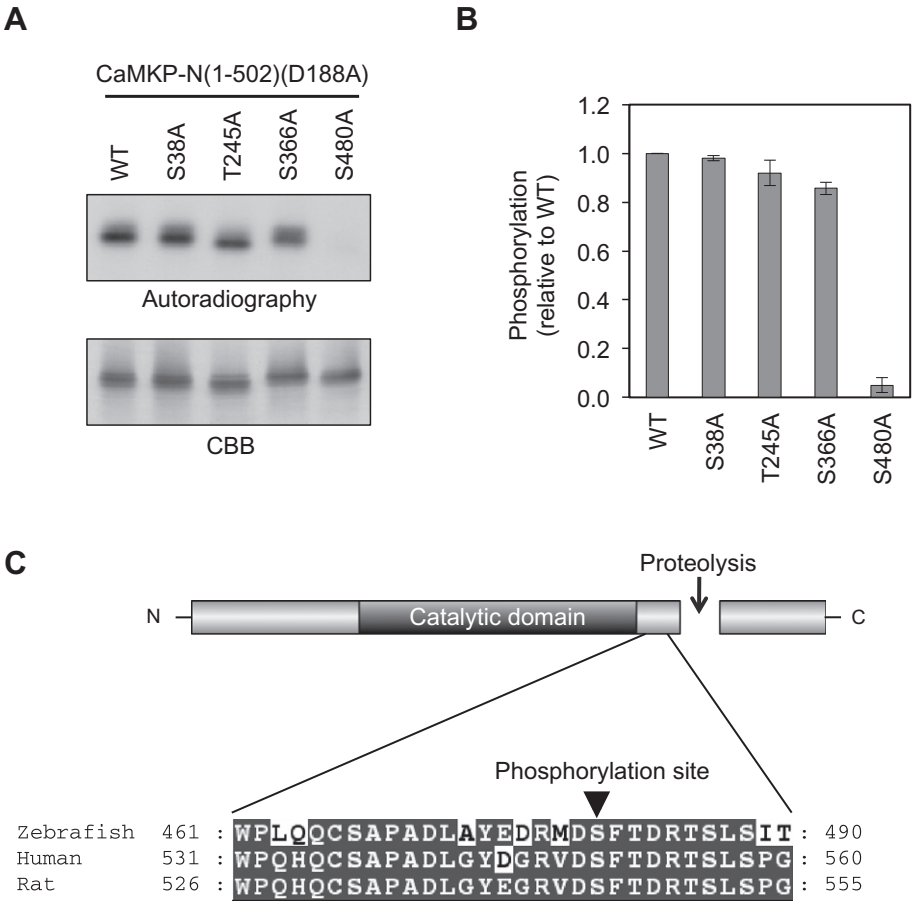

Fig. 3. Identification of the phosphorylation site of zCaMKP-N by CaMKI. (A, B) Point mutants of zCaMKP-N(1-502) (200 ng) were phosphorylated by phospho-CaMKI (2 ng) in a standard phosphorylation mixture (10 μl) containing 100 μM [γ-^{32}P]ATP at 30 °C for 30 min. The phosphorylated proteins were resolved by SDS–PAGE using a 10% gel and detected by autoradiography (A). The radioactive signals were quantified with a Bio-imaging Analyzer BAS1800 (Fuji Film) (B). (C) Schematic illustration of the primary structure of CaMKP-N. The phosphorylation site and the putative cleavage site by endogenous protease are shown by an arrowhead and an arrow, respectively.

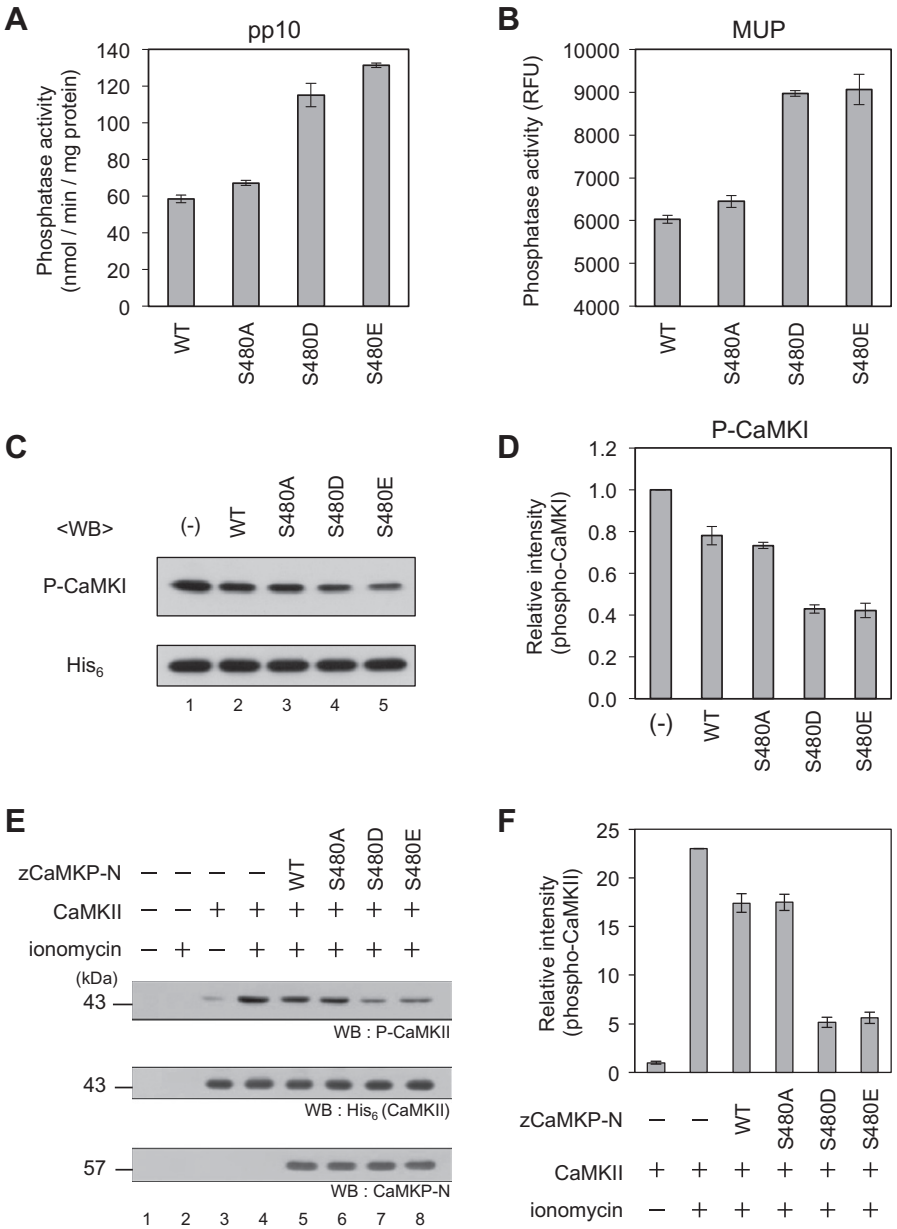

Fig. 4. Phosphatase activities of phosphorylation-mimic mutants of zCaMKP-N. (A) The purified zCaMKP-N point mutants (4 μg/ml) were assayed using 20 μM pp10 as a substrate in the presence of 2 mM $MnCl_2$ in a standard reaction mixture. The inorganic phosphate released into the reaction mixture was determined as described in Section 2. (B) zCaMKP-N and its point mutants (3 μg) were assayed at 30 °C for 10 min using 25 μM MUP as a substrate as described. (C) The phosphatase activities of zCaMKP-N and its point mutants were determined using phospho-CaMKI as a substrate. Dephosphorylation of CaMKI was monitored by Western blotting with an anti-phospho-CaMKI antibody. (D) Phospho-CaMKI (C, upper panel) was quantified by Scion Image software. Data are means ± SD from three independent determinations. (E) Neuro2a cells transfected with pcDNA-zCaMKP-N(1-502, WT and point mutants) and pcDNA-CaMKII were cultured for 24 h. Subsequently, the cells were cultured in serum-free medium for 6 h and stimulated with 1 μM ionomycin for 10 min. The cells were lysed with SDS–PAGE sample buffer and analyzed by Western blotting with anti-phospho-CaMKII, anti-His_6 (for the detection of CaMKII) and anti-CaMKP-N antibodies. (F) Phospho-CaMKII (E, upper panel) was quantified by Scion Image software. Data are means ± SD of the relative intensities to that of lane 3 obtained from three independent experiments.

Table 1
Kinetic analysis of CaMKP-N and its phosphorylation-mimic mutants.

CaMKP-N(1-502)	K_m (μM)	V_{max} (nmol/min/mg)
WT	3.54 ± 0.08	82 ± 2.08
S480D	4.72 ± 0.01	141 ± 5.56
S480E	4.78 ± 0.02	151 ± 4.04

Reactions were performed as described in Section 2 using pp10 as a substrate. Values are means ± SEM of three independent experiments.

ted under the same conditions, autophosphorylation of CaMKII was further attenuated (lanes 7 and 8). Quantitative analysis of immunoreactive bands was carried out using Scion Image software and summarized in Fig. 4F. These data suggest that zCaMKP-N is activated by CaMKI *via* phosphorylation at Ser-480 both *in vitro* and *in vivo*.

4. Discussion

We have been trying to study on the regulation of CaMKP-N since we found the phosphatase in human cDNA database using the sequence of rat CaMKP as a query [5]. Despite considerable research efforts, difficulty in expression of human CaMKP-N in a sufficient quantity and purity has hampered biochemical charac-

terization of this enzyme. To circumvent this problem, as well as to further elucidate its physiological significance at whole-body level, we chose zCaMKP-N as a model, and have extensively studied about biochemical and physiological properties of zCaMKP-N [13]. However, little is known about reversible regulation of zCaMKP-N. In our previous paper, we reported that zCaMKP-N changed its subcellular localization by proteolytic processing via proteasome to dephosphorylate phospho-CaMKI in cytosol, with its phosphatase activity being activated [9]. Since CaMKI is a multifunctional protein kinase that phosphorylates various protein substrates, we supposed that zCaMKP-N might be directly phosphorylated by CaMKI. In this study, we showed that CaMKI actually phosphorylated zCaMKP-N to activate its phosphatase activity, and identified the phosphorylation site as Ser-480. This is the first report on a regulatory mechanism of CaMKP-N by covalent modification. It should be noted that the amino acid sequence around Ser-480 of zCaMKP-N is highly conserved among zebrafish, human, and rat homologs. Mammalian CaMKP-N may also be activated by CaMKI-catalyzed phosphorylation at the Ser residue corresponding to Ser-480 of zCaMKP-N.

Kinetic analysis showed that substitution of Ser-480 with Asp or Glu, which mimics phosphorylation of the residue, increased V_{max} values without significantly affecting K_m values, suggesting that the phosphorylation at this site enhanced catalytic turnover of the enzyme (Table 1). Since Ser-480 is located within the C-terminal domain, not the catalytic domain, some conformational change induced by the phosphorylation might be involved in the mechanism of activation.

Although it is well known that a variety of protein phosphatases are regulated by phosphorylation [21–24], only a limited number of studies have been carried out about regulation of PPM family phosphatases by phosphorylation. In our earlier studies, we reported that CaMKP is phosphorylated and activated by the active catalytic fragment of CaMKII [7]. Awano et al. showed that c-Jun N-terminal kinase phosphorylates PP2Cζ to attenuate its phosphatase activity [25]. Here, we report that zCaMKP-N is phosphorylated and activated by CaMKI. Taken together, reversible phosphorylation is likely to be a fundamental mechanism to properly regulate phosphatase activities of PPM family phosphatases as well as protein phosphatases of other families.

It is of great interest that expression of phospho-mimics at Ser-480 having enhanced phosphatase activity significantly abrogated autophosphorylation of CaMKII, because this suggests that CaMKI-catalyzed phosphorylation of CaMKP-N suppresses generation of Ca^{2+}/CaM-independent autonomous activity of CaMKII. In other words, activation of CaMKI could inhibit the autonomous activity of CaMKII through activation of CaMKP-N. Originally, CaMKP-N was assumed to be localized only in the nucleus, but later studies including ours revealed that it undergoes proteolytic processing to be activated and localized in cytosol [9,26]. The cytosolic CaMKP-N fragment of which the C-terminal region is truncated could be more readily phosphorylated by CaMKI in cytosol to be activated. The activated CaMKP-N would dephosphorylate active CaMKI to downregulate it as a feedback mechanism, as well as abrogate autonomous CaMKII activity to attenuate signals propagated through pathways in which CaMKII is involved. Activation of CaMKP-N by CaMKI, reported here, might be a novel mechanism to modulate subtle balance between signaling pathways mediated by CaMKI and CaMKII.

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